An Intimate Dining – Nutritional Interactions between Obligate Intracellular Parasites and Host Cells

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SUMMARY

The protozoan phylum apicomplexa comprises nearly 6000 parasitic species, many of which are of significant medical and veterinary importance. Most apicomplexans have adapted to obligate intracellular parasitism in a wide range of organisms, including animals and humans. Some notable members of the phylum include *Toxoplasma*, *Plasmodium* and *Eimeria* species, which collectively impose substantial healthcare and socioeconomic burden worldwide. This study focused on three representative parasites, namely *Toxoplasma gondii*, *Eimeria falciformis* and *Plasmodium berghei*, all of which infect a common and well-established model host organism (*i.e.* mouse), but have diverged from each other considerably with respect to the target host cells, persistence and reproduction behavior. For example, *T. gondii* can infect and replicate in most nucleated cells, whereas *P. berghei* and *E. falciformis* are specific to hepatic/red blood cells and intestinal epithelial cells, respectively. Unlike *Plasmodium* and *Eimeria* species causing only acute disease, *T. gondii* can also inflict chronic infection. Moreover, *Toxoplasma* can bypass the sexual phase for inter-host transmission; in contrast, its peers must undergo alternating asexual and sexual growth for the natural transmission. Last but not least, *T. gondii* and *P. berghei* display only asexual growth in mouse, and require a second host, feline and mosquito, respectively, for sexual reproduction, while *E. falciformis* assumes asexual as well as sexual development in a single host. Hence, these parasites together enable a fairly inclusive study of the apicomplexan biology.

A successful intracellular parasite must be able to access host resources and allocate them efficiently to satisfy its own cellular demands irrespective of intracellular niche or lifecycle stage. Apicomplexan parasites undertake a complex development, which involves genome replication, organelle biogenesis and the zoite assembly within other eukaryotic cells (*i.e.* a eukaryote inside a eukaryote). Most developmental stages of these pathogens intimately associate with host cells, involving a metabolic crosstalk between the two entwined entities. A germane understanding of such interactions is vital to appreciate the evolution of parasites. In a nutshell, this work aimed to determine the design of metabolic networks in indicated parasites and the impact of metabolism on growth, pathogenesis and adaptation in discrete nutritional milieus. Our approach blended bottom-up methods of biochemistry, reverse genetics, cell biology and optogenetics with the top-down lipidomics, metabolomics and transcriptomics to address the following major premises:

- Comparative design of the selected metabolic networks in aforementioned parasites
- Nutritional plasticity underlying the parasite survival in variable environments
- Subversion or exploitation of host metabolism by intracellular parasites
- Stage-specific rewiring of parasite metabolism during asexual reproduction
- Identification and endorsement of potential anti-parasitic drug targets

In a series of work, we have revealed that the tachyzoite stage (fast-replicating acute stage) of *T. gondii* is metabolically very active and rather autonomous in making macromolecules, as well as occasionally resilient to nutritional perturbations. Tachyzoites display a surprising plasticity in the central carbon metabolism that ensures their survival even in the absence of glucose. We identified glutamine and acetate as the two other major carbon sources, which support glucose-independent growth of tachyzoites. In particular, glutamine-fueled gluconeogenesis becomes critical in tachyzoites with impaired glycolysis. The work also illustrated a hitherto unknown metabolic convergence between proliferating parasites and cancer cells. In contrast to *T. gondii*, *P. berghei* is strictly dependent on the import and glycolytic catabolism of host-derived glucose for its

entire lifecycle even though the parasite is competent in utilizing glutamine and acetate. Inability of *P. berghei* to survive without glucose is due to absence of glutamine-powered gluconeogenesis.

In additional work, we show that *T. gondii* and *E. falciformis* harbor a nearly complete enzymatic machinery to make major phospholipids and thus seem to be fairly autonomous of respective host cell. *Toxoplasma* displays a flexible membrane biogenesis, which along with malleable carbon flux partly explains its promiscuous growth in assorted host milieus. Our data also demonstrate significant and unexpected divergence in several aspects of lipid biogenesis, not only with respect to mammalian hosts but also within the phylum apicomplexa. We observed that tachyzoites of *T. gondii* and the sporozoite stage of *E. falciformis* express exclusive sphingolipids, ethanolamine-phosphorylceramide and inositol-phosphorylceramide, respectively. Equally, we identified a novel and major phospholipid expressed in *T. gondii* and *E. falciformis* (but not in *Plasmodium* and mammalian cells), termed phosphatidylthreonine, which has evolved from an otherwise-universal lipid, phosphatidylserine. A tachyzoite mutant lacking phosphatidylthreonine exhibits impaired calcium homeostasis, which in turn compromises the lytic cycle and virulence. Notably, such a metabolically attenuated parasite strain can be used as a *vaccine* to prevent toxoplasmosis in a mouse model. The natural expression and specialized functions of phosphatidylthreonine and indicated sphingolipids signify adaptive divergence of membrane lipids in parasitic protists, which offers an attractive intervention strategy to selectively inhibit the parasite reproduction.

Our active engagement with *E. falciformis* has also identified the host determinants of infection *in vivo*. We have discovered a retinue of IFNγ-regulated host factors, *e.g.*, tryptophan catabolism, immunity-related GTPases and chemokine signaling, some of which are protective, whereas others are evaded or even exploited by the parasite. This study found how mouse IFNγ-signaling plays opposing roles in promoting and demoting the *Eimeria* development. Especially, induction of the first enzyme of tryptophan catabolism (indoleamine 2,3-dioxygenase) in the mouse caecum is needed for efficient *in vivo* growth of *E. falciformis*. Likewise, we show a requirement of host cFos (a proto-oncogenic master transcription factor) for *in vitro* growth of *E. falciformis* and *T. gondii*. Finally, as reckoned necessary to resolve our above-mentioned paradigms, we established new or improved methods. For example, we applied optogenetics to regulate cyclic nucleotide-directed signaling in *T. gondii*, which enabled an otherwise-challenging induction of the parasite cAMP in a specific, reversible and spatiotemporal manner without perturbing the surrounding host cells. Using this tool, we determined a requisite of cAMP for the parasite differentiation and associated gene expression. Besides, by employing an optogenetic sensor (gene-encoded calcium indicator), we discovered the importance of phosphatidylthreonine in regulating the cytosolic calcium pool in intracellular tachyzoites. In brief, optogenetically-modified strains allow a more systematic dissection and detection of the signaling cascades in intracellular parasites, which has not been so feasible when using chemical modulators and probes. Our initial application of light-responsive actuators and sensors to illuminate the parasite biology is expected to lead a wider deployment of optogenetics in infection research.

ZUSAMMENFASSUNG

Das zu den Protozoen gehörende Phylum der Apicomplexa umfasst nahezu 6000 Parasitenarten, von denen einige von medizinischer und veterinärmedizinischer Bedeutung sind. Die meisten Apicomplexa haben sich an eine obligat intrazelluläre Lebensweise angepasst und infizieren verschiedenste Tiere und den Menschen. Zu den bedeutendsten Vertretern der Apicomplexa zählen *Toxoplasma*, *Plasmodium* und *Eimeria*, welche eine wesentliche Belastung für das Gesundheitswesen und Sozioökonomie darstellen. In dieser Arbeit lag der Schwerpunkt auf den drei repräsentativen Organismen *Toxoplasma gondii*, *Eimeria falciformis* und *Plasmodium berghei*, welche sich alle in einem etablierten Wirt (der Maus) reproduzieren, sich allerdings hinsichtlich ihrer Wirtszellen, Persistenz sowie in ihrem Reproduktionsverhalten deutlich unterscheiden. *T. gondii* infiziert beispielsweise nahezu alle kernhaltigen Zellen, während *P. berghei* und *E. falciformis* spezifisch Leber- und Blutzellen bzw. Darmepithel befallen. *Plasmodium* und *Eimeria* verursachen ausschließlich akute Erkrankungen, eine Infektion mit *Toxoplasma* kann auch chronisch verlaufen. Im Gegensatz zu *Plasmodium* und *Eimeria*, die den Wechsel zwischen sexueller und asexueller Vermehrung bei der natürlichen zwischenwirtlichen Transmission durchlaufen müssen, kann *Toxoplasma* die sexuelle Phase umgehen. Letztlich vermehren sich *T. gondii* und *P. berghei* in der Maus ausschließlich asexuell und benötigen einen zweiten Wirt (Katzen bzw. Moskitos) für die sexuelle Reproduktion, während *E. falciformis* sowohl die sexuelle als auch die asexuelle Phase in der Maus durchläuft. Somit ermöglichen die genannten Parasiten eine umfassende Untersuchung der Biologie der Apicomplexa.

Ein erfolgreicher intrazellulärer Parasit muss in der Lage sein auf Ressourcen des Wirts zuzugreifen und diese für den eigenen zellulären Bedarf zu nutzen. Apicomplexe Parasiten durchleben eine komplexe Entwicklung einschließlich Genomreplikation, Organellen- und Tochterzellbildung in einer anderen eukaryotischen Zelle. Die meisten Entwicklungsstufen dieser Pathogene sind sehr eng mit der Wirtszelle assoziiert, was auch metabolische Wechselwirkungen beinhaltet. Das Verständnis dieser Interaktionen ist unerlässlich, um die Evolution von Parasiten zu ergründen. Grundsätzlich war das Ziel dieser Arbeit, die metabolischen Netzwerke der genannten Parasiten zu eruieren und den Einfluss des Metabolismus auf Wachstum, Pathogenese und Adaption in verschiedenen Nährstoffumgebungen zu untersuchen. Unsere Vorgehensweise verband biochemische, reverse-genetische, zellbiologische und optogenetische Bottom-Up-Methoden mit Top-Down-Methoden wie Lipidomics, Metabolomics und Transcriptomics um folgende Prämissen anzugehen:

- Vergleichender Entwurf der metabolischen Netzwerke in den obengenannten Parasiten
- Nährstoff-Plastizität für die Überlebensfähigkeit des Parasiten in verschiedenen Milieus
- Umregulierung oder Ausbeutung des Wirtsmetabolismus durch intrazelluläre Parasiten
- Stadien-spezifische Regulation des Metabolismus während der asexuellen Reproduktion
- Identifizierung und Validierung potentieller anti-parasitischer Wirkstoffe

In unserer bisherigen Arbeit haben wir gezeigt, dass das Tachyzoiten-Stadium (akute Form der Infektion) von *T. gondii* metabolisch sehr aktiv, weitgehend autonom in der Biogenese von zellulären Makromolekülen und mitunter sehr widerstandsfähig bei variierender Nährstoffverfügbarkeit ist. Tachyzoiten zeigen eine enorme metabolische Plastizität im zentralen Kohlenstoffmetabolismus, welche sogar ein Überleben ohne Glukose ermöglicht. Weiterhin konnten wir nachweisen, dass Glutamin und Acetat zwei weitere bedeutende Kohlenstoffquellen neben Glucose darstellen. Vor allem die glutamingetriebene Gluconeogenese wirkt sich entscheidend auf Tachyzoiten mit einer beeinträchtigten Glykolyse aus. Diese Untersuchungen zeigten eine bisher unbekannte metabolische Konvergenz zwischen proliferierenden Parasiten und Krebszellen. Im Vergleich dazu ist *P. berghei*

darauf angewiesen Zucker des Wirts in seinem gesamten Lebenszyklus zu importieren, weil die Gluconeogenese fehlt, obwohl der Parasit auch fähig ist Glutamin und Acetat zu nutzen.

Wir zeigten zudem, dass sowohl *T. gondii* als auch *E. falciformis* eine nahezu vollständige Enzymkette für die Biosynthese der wichtigsten Phospholipide beherbergen und somit ziemlich unabhängig von ihrer jeweiligen Wirtszelle zu sein scheinen. *Toxoplasma* zeigt eine flexible Membranbiogenese, welche zusammen mit einem variablen Kohlenstoffffluss zum Teil seine Wachstumsfähigkeit in verschiedenen Nährstoffmilieus erklärt. Darüber hinaus demonstrieren unsere Ergebnisse eine erhebliche sowie unerwartete Divergenz hinsichtlich der Stoffwechselwege zur Membranbiogenese - sowohl in Bezug auf den Säugerwirt aber vor allem auch innerhalb der Apicomplexa. Wir konnten beobachten, dass Tachyzoiten von *T. gondii* und Sporozoiten von *E. falciformis* exklusive Sphingolipide, Ethanolamin-Phosphorylceramid bzw. Inositol-Phosphorylceramid, bilden. Darüber hinaus konnten wir ein bisher unbekanntes Phospholipid Phosphatidylthreonin identifizieren, welches in *T. gondii* und *E. falciformis* exprimiert ist (jedoch nicht in *Plasmodium* und Säugern) und sich aus dem anderweitig universellen Phosphatidylserin entwickelt hat. Die Tachyzoit-Mutante, der dieses exklusive Lipid fehlt, zeigt eine gestörte Kalziumhomöostase, welche wiederum den lytischen Zyklus sowie die Virulenz des Parasiten beeinträchtigt. Bemerkenswerterweise kann ein solcher metabolisch attenuierter Parasitenstamm als *Impfstoff* verwendet werden und so eine Erkrankung an Toxoplasmose im Mausmodell verhindern. Die Expression und spezialisierten Rollen von Phosphatidylthreonin und der angegebenen Sphingolipide kennzeichnen eine adaptive Divergenz der Membranlipide in parasitischen Protisten, welche einen attraktiven Angriffspunkt für die selektive Inhibierung des Parasiten-Wachstums liefert.

Unsere Untersuchungen an *E. falciformis* waren erfolgreich hinsichtlich der bestimmenden Faktoren in Bezug auf den Wirt *in vivo*. Dabei haben wir eine Reihe IFNγ-regulierter Stoffwechselwege im Wirt, wie beispielsweise den Tryptophan-Katabolismus, mit der Immunität in Zusammenhang stehende GTPasen sowie eine Chemokin-Signaltransduktion, entdeckt, von denen einige eine protektive Wirkung haben, anderen wiederum kann der Parasit entkommen oder diese sogar ausnutzen. Unsere Arbeit zeigt, wie die IFNγ-Signalgebung in der Maus entgegengesetzte Rollen im Fördern und Schaden der Entwicklung von *Eimeria* spielt. Insbesondere ist die Induktion des ersten Enzyms des Tryptophan-Katabolismus (Indoleamin-2,3-Dioxygenase) im Darm der Maus für ein effizientes Wachstum von *E. falciformis* notwendig. Gleichzeitig konnten wir den Bedarf an Wirts-cFos (ein proto-onkogener Haupttranskriptionsfaktor) für die *in vitro*-Entwicklung von *E. falciformis* und *T. gondii* aufzeigen. Schlussendlich etablierten wir neue und verbesserte Methoden, um die genannten Problemstellungen anzugehen. So nutzten wir beispielsweise optogenetische Methoden, um die Signalkaskaden zyklischer Nukleotide in *T. gondii* in einer spezifischen, reversiblen und raum-zeitlich definierten Weise zu manipulieren, ohne die Wirtszelle zu beeinflussen, was sich bisher als schwierig erwies. Mit dieser Methode konnten wir den Bedarf von cAMP für die Stadien-Differenzierung und assoziierte Genexpression aufzeigen. Weiterhin haben wir mit Hilfe dieses optogenetischen Sensors (ein gencodierter Kalziumindikator) eine Funktion von Phosphatidylthreonin bei der Regulation des zytosolischen Kalziums in intrazellulären Tachyzoiten entdeckt. Kurz gesagt ermöglichen solche optogenetisch veränderten Stämme eine systematischere Aufschlüsselung bzw. Detektion von Signalkaskaden in intrazellulären Parasiten, was bisher allein durch chemische Untersuchungen nicht möglich war. Unsere Anwendung Licht-responsiver Akteure und Sensoren zur näheren Beleuchtung der Biologie der Parasiten wird möglicher Weise eine breitere Anwendung von Optogenetik in der Infektionsbiologie ermöglichen.

ABBREVIATIONS

Abbreviation	Description
ACS	Acetyl CoA synthetase
CCT	CTP:phosphocholine cytidylyltransferase
CDP-DAG	CDP-diacylglycerol
CDS	CDP-DAG synthase
CK	Choline kinase
cNMP	Cyclic nucleotide monophosphate (cAMP, cGMP)
CPT	Choline phosphotransferase
DAG	Diacylglycerol
DG	Dense granule
DLC	Dynein light chain
DME	Dimethylethanolamine
ECT	CTP:phosphoethanolamine cytidylyltransferase
EK	Ethanolamine kinase
EPC	Ethanolamine-phosphorylceramide
EPT	Ethanolamine phosphotransferase
ER	Endoplasmic reticulum
FAS/FAE	Fatty acid synthase / Fatty acid elongase
FBP	Fructose 1,6-bisphosphatase
G3P	Glycerol-3-phosphate
GBP	Guanylate-binding protein
GRA	Granule (proteins secreted by dense granules)
GT1	Glucose transporter 1
HT1	Hexose transporter 1
IDO	Indoleamine 2,3-dioxygenase
IPC	Inositol-phosphorylceramide
IRG	Immunity-related GTPase
LDL	Low density lipoprotein
MIC	Microneme (or proteins secreted by micronemes)
MNN	Membranous nanotubular network
PDME	Phosphatidyldimethylethanolamine
PECK	Phosphoenolpyruvate carboxykinase
PIS	Phosphatidylinositol synthase
PKA	Proteins kinase A (cAMP-dependent)
PKG	Protein kinase G (cGMP-dependent)
PSD	Phosphatidylserine decarboxylase
PSS	Phosphatidylserine synthase
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
PtdIns	Phosphatidylinositol
PtdOH	Phosphatidic acid
PtdSer	Phosphatidylserine
PtdThr	Phosphatidylthreonine
PTS	Phosphatidylthreonine synthase
PV/PVM	Parasitophorous vacuole / PV membrane
RON	Rhoptry neck (proteins located in the rhoptry-neck region)
ROP	Rhoptry or proteins located in the rhoptry-bulb region
TVN	Tubulovesicular network

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1 INTRODUCTION

1.1 Parasitism and its impact in nature

The organisms in an ecosystem interact in different ways, which may exert positive, neutral or negative impact on each other. The term symbiosis usually refers to these interactions between two (or more) organisms living together with some sort of feeding relationship involved [1,2] (Fig 1). The three commonly observed symbiotic interactions are mutualism, in which both organisms benefit; commensalism, where one of the two partners benefits, while the other organism is not harmed; and parasitism, where one (parasite) profits at the expense of other (host) organism [1,2]. Parasites typically depend on host resources for the survival. They may live outside (ectoparasite) or inside (endoparasite) a host [3]. An endoparasite spends significant part of its life inhabiting on tissues (extracellular) or within cells (intracellular) of a host organism.

Fig 1: *Emoticon illustration of three major symbiotic relationships observed in nature*. Unlike mutual and commensal interactions between the two organisms, in which both or at least one partner gains and none are harmed, a parasitic relationship involves an inverse association.

Although the concept of parasitism and the term parasite apply unambiguously to many cases in nature, *e.g.*, bacteria and viruses, they generally entail to eukaryotic pathogens of protozoan and metazoan origins. The kingdom protozoa comprises more than 40,000 known living species, of which about 25,000 species occur as free-living, whereas the remaining are adapted to parasitic life [4,5]. The latter group includes at least 250 amoebae, 1800 flagellate, 2500 ciliate and 6000 sporozoan (apicomplexan) species. These parasitic organisms together impose significant burden on our healthcare systems worldwide. Not only do they affect infected hosts by altering growth, behavior, nutritional status, reproductive abilities and mortality, but also shape our ecosystem by influencing trophic interactions, food webs and biodiversity [6].

1.2 Obligate intracellular (apicomplexan) parasites

The phylum apicomplexa contains the bulk of what was once termed as the sporozoa, a group of spore-forming parasitic protozoans lacking pseudopods and cilia altogether, and flagella from the most lifecycle stages [7,8]. They have likely evolved from a photosynthetic algal ancestor [9]. A transition from the free-living to parasitic lifestyle is supported by the existence of closely related predator (*colpodellids*) and symbiont (*Nephromyces*) of marine organisms [10,11]. The apicomplexan members have adapted to become mostly obligate intracellular parasites with complex lifecycles, which often involve multiple types of cells, tissues and host organisms. Indeed, these parasites are

ubiquitously distributed across the animal kingdom. They can be grouped based on phylogenetic relationships and tissue specificities [12,13] (Fig 2). Hematozoans infect blood cells of a vertebrate host to achieve the asexual reproduction and require a blood-feeding invertebrate host for sexual reproduction and transmission. The well-known hematozoans include *Plasmodium* species causing malaria, along with *Babesia* and *Theileria* species, which infect cattle [3]. The coccidian parasites, forming the largest group in the phylum, typically infect intestinal and extra-intestinal tissues and spread through the fecal-oral route. Some of the prevalent coccidian genera include *Toxoplasma*, *Sarcocystis*, *Eimeria*, *Isospora* and *hepatozoon* that infect animals and/or humans [14]. In particular, a single species of *Toxoplasma* (*T. gondii*) is capable of infecting nearly all warm-blooded organisms [15]. On the other hand, individual species of the genus *Eimeria* (>1800 species) are highly host-specific and cause diarrhea in a variety of animals, most notably in the poultry [16]. Gregarine is a distant subgroup, which consists of primarily extracellular pathogens of invertebrates [17], but also includes clinically relevant epicellular parasites, such as *Cryptosporidium* species [18].

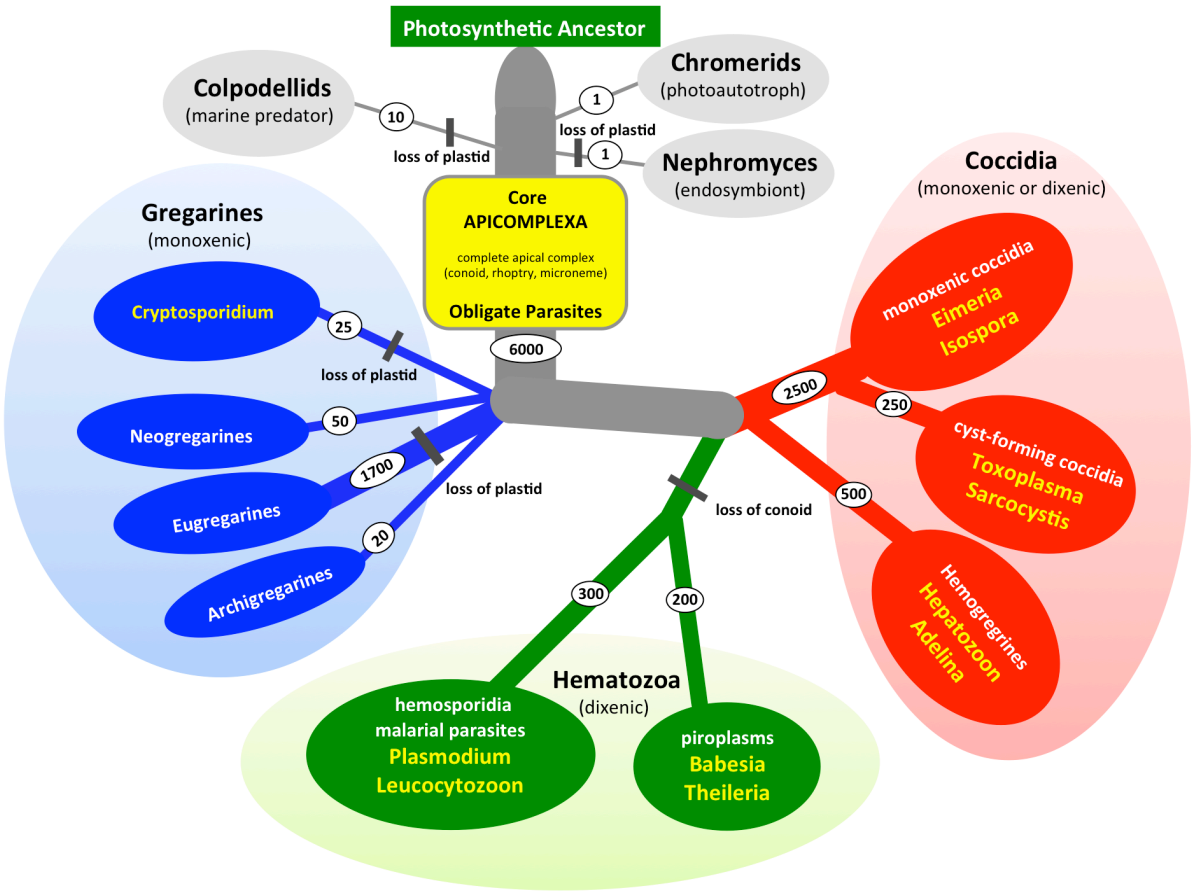

Fig 2: *Phylogenetic tree of apicomplexan parasites*. Only representative parasite groups (*e.g.*, hematozoa, coccidia and gregarines) are depicted. Numbers and thickness of branches indicate the approximate diversity (*i.e.* known species) in each clade. The hallmark features and common parasite members are also mentioned. Apicomplexans have evolved from a photosynthetic ancestor (*chromerids*). They are closely related to free-living predators (*colpodellids*) and endosymbionts (*Nephromyces*). *Cryptosporidium* likely emerged from within gregarines. Image has been redrawn from the reference [13] to emphasize the context.

1.3 Lifecycle of apicomplexan parasites

The natural lifecycle of apicomplexan parasites comprises both asexual and sexual reproduction gyrating often between the primary (*i.e.* definitive or sexual) and secondary (*i.e.* intermediate or asexual) hosts [3,14] (Fig 3). *Toxoplasma* and *Plasmodium* species complete their entire development in two hosts (dixenic), while *Eimeria* and *Cryptosporidium* species require only one host (monoxenic) (Fig 2). *Plasmodium*, *Eimeria* and *Cryptosporidium* species are very specific to the host, tissue and cell types they infect; conversely, *T. gondii* is quite promiscuous with little to no regard to any of these. Infective stages of the apicomplexan parasites are termed as zoites that are formed after sexual (sporozoite) or after asexual (merozoite, tachyzoite, bradyzoite *etc.*) reproduction [14,19]. Asexual growth of zoites is followed by sexual development producing male and female gametes that fuse together to form a zygote, which later develops into an oocyst. The oocyst stage undergoes the process of sporogony producing the sporozoite stage and thereby completing the lifecycle.

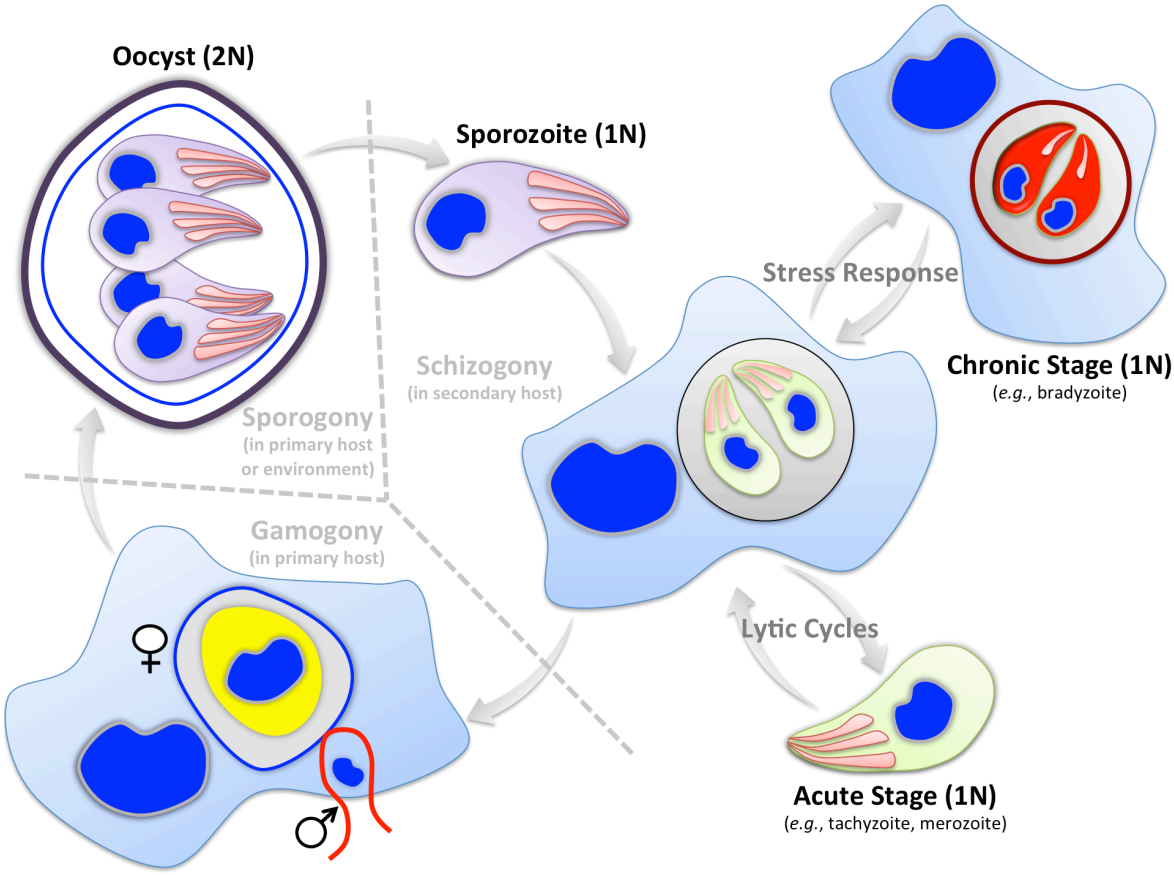

Fig 3: *Simplified lifecycle of apicomplexan parasites.* Only the most generic type lifecycle and underlying stages are depicted. Many deviations are seen in nature, particularly with respect to the asexual development. As illustrated, lifecycle is completed either in one or two hosts (dotted line). Most developmental stages of the apicomplexan parasites are haploid except for the oocyst, which is diploid. Inter-host transmission usually requires the entire cycle, which initiates with the formation of sporozoites within oocysts *via* sporogony (a type of meiosis). Asexual reproduction occurs generally by schizogony (a type of mitosis). Some parasites (*e.g.*, *Toxoplasma*, *Neospora*) can also form dormant or chronic stages in response to physicochemical stress.

Asexual reproduction of *Plasmodium* in its intermediate host begins with the injection of infective sporozoites by the primary host, mosquito [20]. Sporozoites infect and reproduce in hepatocytes, which eventually release thousands of merozoites into bloodstream. Merozoites replicate in red blood cells by multiple rounds of erythrocytic schizogony. A fraction of merozoites differentiates into gametes, which undergo sexual commitment to ultimately yield the sporulated oocysts in the mosquito host. Lifecycle of *Eimeria* species initiates with the ingestion of sporulated oocysts from the environment, ensued by infection of intestinal epithelium with freed sporozoites, schizogony, gamogony and oocyst development, all occurring in one host [14]. In the case of *Toxoplasma*, the secondary hosts get infected either by ingesting oocysts containing sporozoites from environment, or by tissue cysts harboring bradyzoites (present in infested meat), both of which differentiate into tachyzoites in intestinal epithelium [21]. Tachyzoites undertake lytic cycles causing tissue necrosis and then spread to immunoprivileged sites (brain, eyes, muscles *etc.*) before forming bradyzoites. Bradyzoites can start either schizogony producing merozoites and then sexual reproduction upon predation of a chronically-infected secondary host by a primary host, or alternatively can resume asexual growth as tachyzoites when ingested by another secondary host [21]. Hence, *Toxoplasma* is unique in its ability to bypass the sexual phase and transmit from asexual to asexual hosts.

1.4 Structure and morphology of an apicomplexan zoite

A plethora of intracellular pathogens have evolved to invade and develop in host cells; however, only few would contest the subcellular complexity observed in apicomplexan zoites. The zoite is a highly polarized cell delimited by the pellicle, a tri-bilayer alveolate-specific structure comprising plasma membrane and inner membrane complex (Fig 4). Underneath the pellicle are the apical and basal complexes, located at the anterior and posterior ends, respectively [22]. The pellicle is associated to the subpellicular microtubule network, which acts as the parasite skeleton [22]. The inner membrane complex extends from the apical to the basal complex, and harbors the gliding machinery to drive the actin-myosin-dependent parasite motility. The parasite body contains a multitude of dedicated organelles, which enable it to perform the sequential tasks of invasion and subsequent development [23,24]. In short, a mature zoite is designed to identify and invade the target host cell, proliferate intracellularly, and often differentiate into the next lifecycle stage [23].

The apical complex is an emblematic feature of the phylum, though its constituents vary among members. It consists of the parasite-specific secretory organelles, microneme and rhoptry, and a polar ring (Fig 4). In coccidians, the apical complex also comprises the conoid – a cylinder-like structure of spirally-arranged tubulin – that is extended during gliding motility and invasion. The parasite contains many exclusive organelles (rhoptries, micronemes, dense granules, apicoplast, plant-like vacuole, acidocalcisomes, exonemes, refractile bodies) as well as the typical eukaryotic organelles (nucleus, mitochondrion, centriole, Golgi body, endoplasmic reticulum) [14,19]. Some of these are parasite or even stage-specific, *e.g.*, plant-like vacuole in *Toxoplasma* [25], food vacuole and exonemes in *Plasmodium* [26,27] and refractile bodies in *Eimeria* [28]. Differences in the organelle makeup of each parasite or stage generally correlate with the specialized behavior and adaptation to respective intracellular niche [29,30]. The apicoplast (enclosed by four membranes)

is in fact a vestigial chloroplast of algal origin acquired by secondary endosymbiosis [31,32]. It is present in most members of the phylum except for gregarines (Fig 2). The apicoplast has lost the ability to perform photosynthesis, but retained certain important metabolic pathways [33,34].

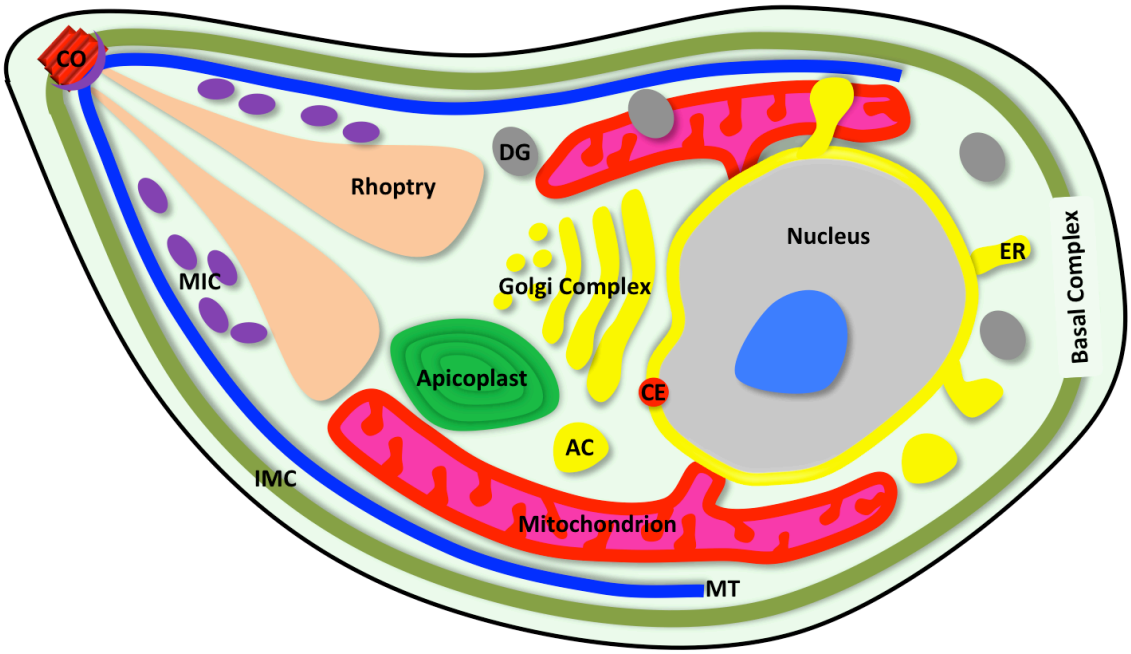

Fig 4: *Schematic illustration of a zoite*. Only the organelles conserved across most parasites are depicted. Not every structure shown here occurs in all apicomplexan parasites. The morphology of individual zoites may differ from the depiction, which signifies the tachyzoite stage of *T. gondii* (sporozoite, slender elongated; merozoite, oval to slender elongated; tachyzoite, crescent; bradyzoite, crescent to elongated; ookinete, oval-elongated). To see a merozoite of *P. falciparum* (within a red blood cell), please refer to Figure 7. AC, acidocalcisome; CE, centrosome; CO, conoid; DG, dense granule; ER, endoplasmic reticulum; IMC, inner membrane complex; MIC, microneme; MT, microtubule

1.5 Asexual reproduction in apicomplexan parasites

The pathology caused by individual parasites is due to multiple rounds of asexual reproduction, often leading to host-cell lysis, tissue necrosis and inflammation. Acute infection occurs by serial lytic cycles that involve invasion, replication, parasite egress and subsequent infection of nearby cells (Fig 5). Once attached and apically oriented onto the host cell surface, the parasite invades within a minute, enclosed in a special membranous structure termed the parasitophorous vacuole (PV) [35,36]. Events of invasion and intracellular establishment require an orchestrated secretion of micronemes, rhoptries and dense granules. Secreted proteins enable several functions that include motility, invasion, maturation of the PV, egress and defense against the host-cell insults [30]. Mechanisms of invasion and formation of nascent PV are mostly conserved since these are regulated by a fairly conserved set of micronemal (MIC) and rhoptry-neck (RON) proteins [37–40]. However, the way each parasite manipulates its host differs considerably due to divergence in rhoptry-bulb (ROP) and dense granule (GRA) proteins. The ROP and GRA proteins contrast significantly with respect to their presence, number, localization and function within the phylum.

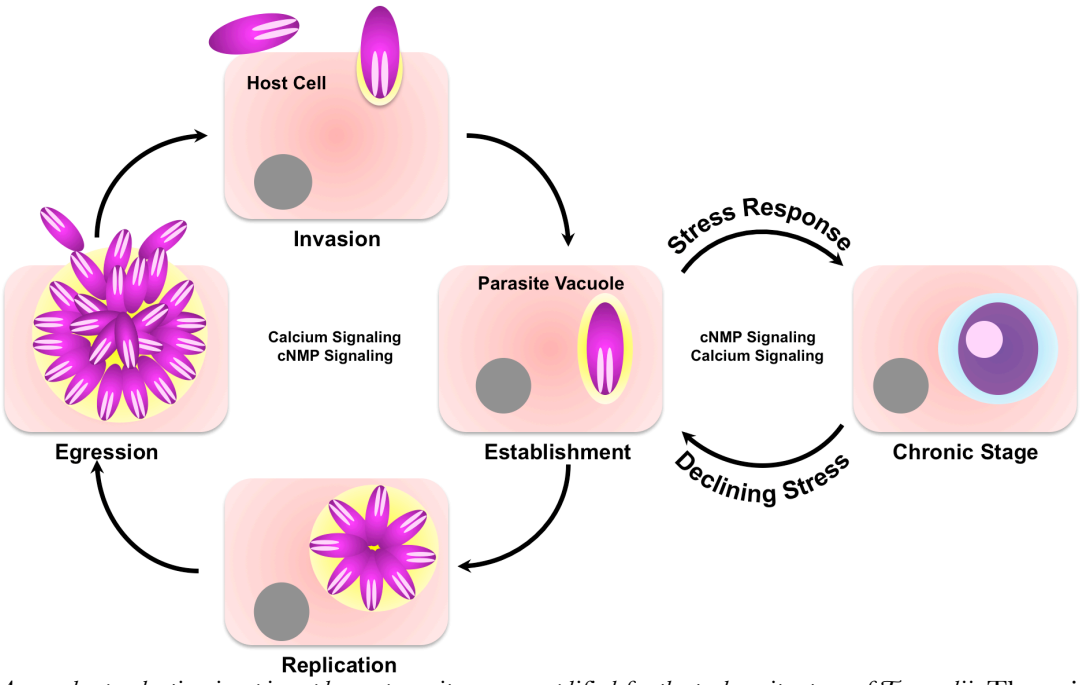

Fig 5: *Asexual reproduction in apicomplexan parasites as exemplified for the tachyzoite stage of T. gondii*. The zoite stage invades the host cell, proliferates to form progeny, which egress to begin a new lytic cycle (acute infection). Sporozoites and bradyzoites differentiate into the next stage during the asexual growth, while tachyzoites and merozoites do not always undergo stage switching. Most zoites divide in a parasitophorous vacuole except for *Theileria*, *Babesia* and *Sarcocystis* species, which escape the nascent vacuole to eventually reside in the cytosol. Physicochemical and immune stresses cause certain parasites, notably *Toxoplasma* and *Neospora*, to assume dormancy as tissue cyst (chronic infection), which may reactivate upon declined stress to initiate the lytic cycle (recrudescence). Calcium and cyclic nucleotides are the two core regulators of the lytic cycle and stage differentiation. Some parasites (*Plasmodium, Babesia*) infect non-nucleated host cells.

Successive phases of intracellular replication ultimately cause parasites to egress by lysing the host cell, which occurs after variable number of cell cycles depending on the parasite and host cell. In most apicomplexans, egress is a signaling-mediated active process that involves timely sensing of the host environment by the parasite [41–43]. Perturbation of ion homeostasis in the dying host cell and activation of calcium- and cGMP-dependent signaling in the parasite are among many other factors governing the gliding motility and subsequent egress and invasion events. Immune response and physicochemical stress can trigger encystation of certain parasites into tissue cysts [21] (Fig 5), which remain latent until reactivated under favorable condition, such as weakened immunity. The apicomplexans have evolved distinct strategies to execute their cell cycles, which are designed to ensure that progeny are mature enough to initiate a new lytic cycle or further development [44] (Fig 6). Merozoites of *Plasmodium*, *Eimeria* and *Toxoplasma* assume schizogony; in which cytokinesis does not instantly follow karyokinesis. Instead, many cycles of DNA replication and nuclear divisions produce a schizont stage prior to the assembly of several zoites. *Toxoplasma* tachyzoites divide by endodyogeny, a variant of schizogony, where karyokinesis and cytokinesis proceed soon after DNA replication. Unlike schizogony, endodyogeny yields only two cells per cycle [44]. Cell division is orchestrated by waves of transcript expression encoding for cell cycle

constituents and regulators. It appears to be regulated globally as well as locally, especially in the schizont stage. Global regulation is exerted by diffusible proteins, such as transcription factors, mitosis-specific kinases and cyclins. Local regulation acts on each nucleus and daughter cells [44].

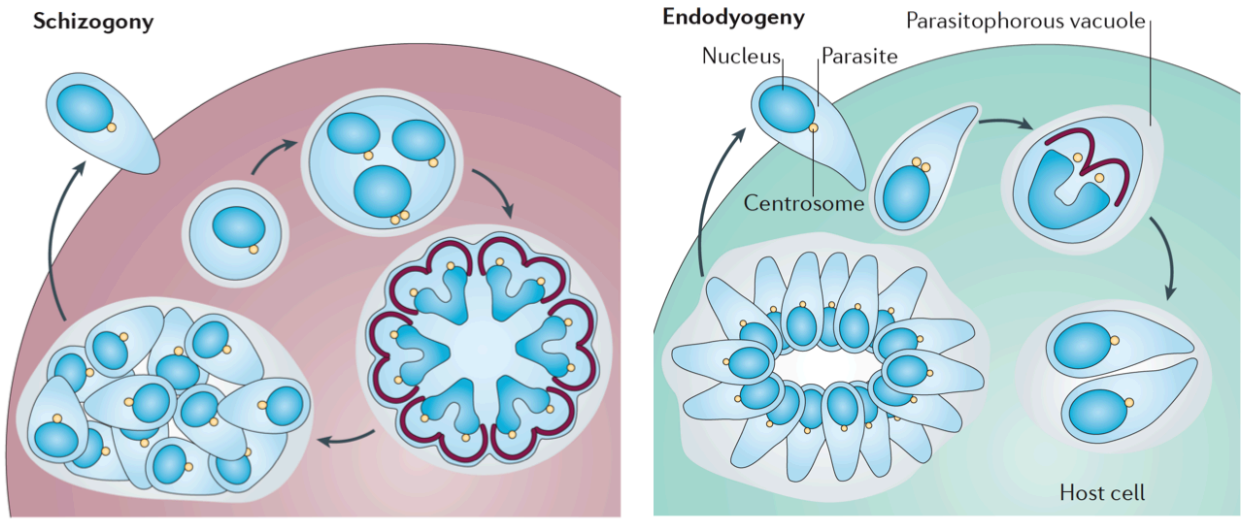

Fig 6: *Cell division in apicomplexan parasites*. Only two usual modes of asexual growth (*Plasmodium*, *Eimeria* and *Toxoplasma*) are shown. *Schizogony*: many rounds of DNA replication and nuclear divisions precede budding of daughter cells at the plasma membrane. *Endodyogeny*: one cycle of DNA replication and nuclear division are immediately followed by budding of two progeny within the mother-cell cytoplasm. Image is adapted from the reference [44]. Centrosome, yellow circles; inner membrane complex, brown; nucleus, blue

1.6 Parasitophorous vacuole and remodeling of infected host cell

Nearly all zoites except for some hematozoan blood stages infect biosynthetically active host cells, which often include even the immune cells (dendritic cells, macrophages, lymphocytes) [45]. The parasites' foremost tasks are therefore to escape lysosomal fusion and cell-intrinsic immunity, and intercept nutrients. To accomplish all these, they extensively manipulate their respective host cell. Individual members differ in relationship with intracellular niches, which is mainly dictated by rhoptry-bulb and dense granule proteins [39,40]. Most parasites develop within a PV, which is initially derived from the host plasma membrane during the process of invasion but significantly modified to safeguard the parasite development [46–49]. The mature PV does not intersect with the endocytic and exocytic traffic and avoids lysosomal acidification. As known in *T. gondii* [47], the PVM forms deep invaginations into the intravacuolar space and long extensions in the host cytosol (Fig 7). The PV of tachyzoites physically tethers with the host endoplasmic reticulum and mitochondria, and recruits cytoskeleton [50]. Interaction of the PVM with the host ER (but not mitochondria) has also been reported in hepatic cells infected with *Plasmodium* sporozoites [51]. Likewise, the merozoite stage induces the formation of Maurer's clefts (flat membrane structures), PVM extensions and cytoskeleton rearrangements in the erythrocyte cytosol along with knob-like structures and new permeation pathway on the surface [52–54] (Fig 7). Many other variations in the PV and host-cell manipulation exist in other apicomplexan parasites [39,45].

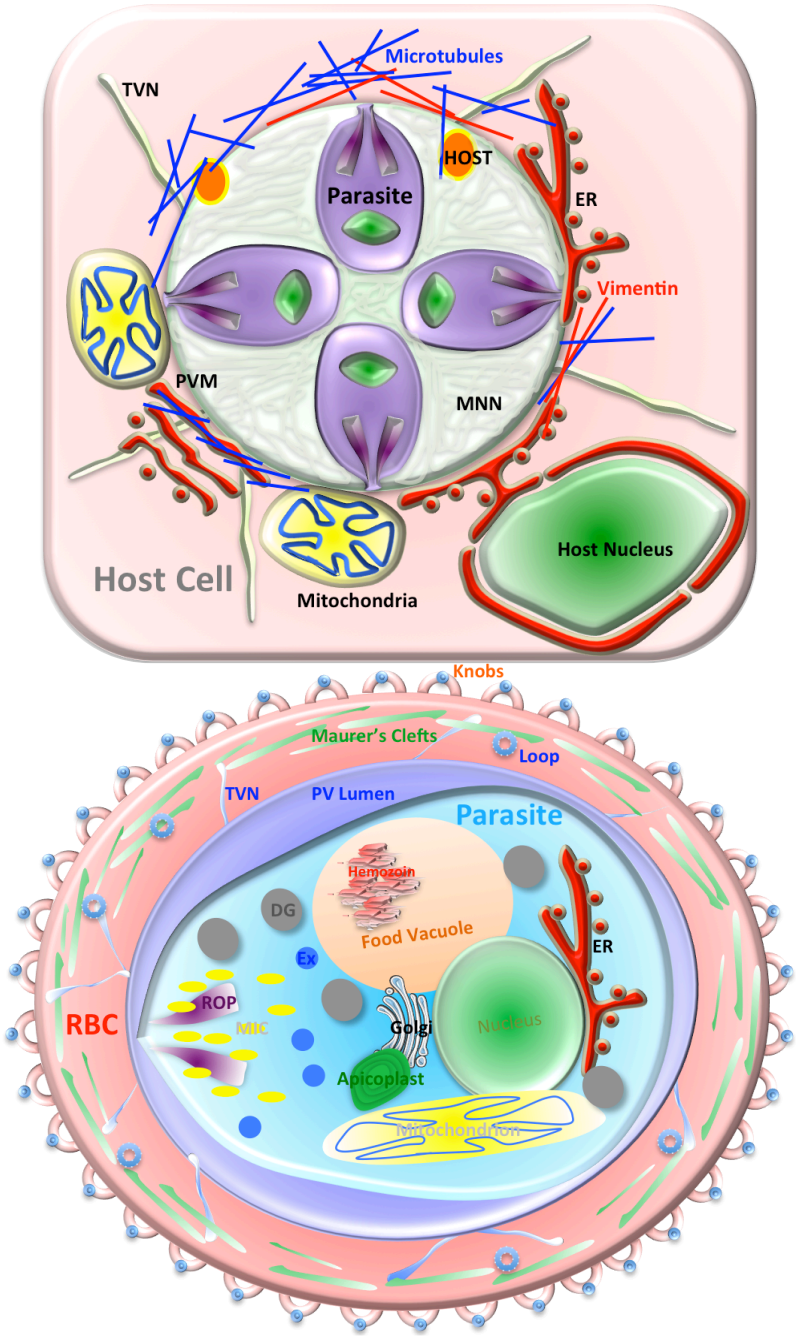

Fig 7: *Remodeling of host cells induced by Toxoplasma and Plasmodium*. Images exemplify only selected aspects. *Top panel*: *Toxoplasma* tachyzoites in a nucleated mammalian cell. Intravacuolar space is decorated with the membranous nanotubular network (MNN). Extensions of PV into host cytosol (tubulovesicular network or TVN) and intravacuolar invaginations of the PVM (*i.e.* Host Organelle Sequestering Tubules; HOST) are also shown. The host-cell ER, mitochondria and cytoskeleton (tubulin, vimentin) become rearranged onto the PVM. *Bottom panel*: A *Plasmodium* merozoite in an erythrocyte. The parasite induces the formation of Maurer's cleft, membrane loops, TVN, knob-like structures (as depicted), as well as cytoskeletal alterations and new permeation pathway (not shown) in the infected cell. Unlike *T. gondii* tachyzoites, where the PV is the main destination for most rhoptry-bulb and dense granule proteins, the PV of *Plasmodium* merozoites constitutes a mandatory transit point for the protein cargo *en route* to host erythrocytes. DG, dense granule; ER, endoplasmic reticulum; Ex, exoneme; MIC, microneme; RBC, red blood cell; ROP, rhoptry

In addition to specified morphological alterations, infection of host cells with individual parasites also results into transcriptomic and proteomic modulation of distinct pathways, such as protein synthesis, metabolism, immune signaling, membrane trafficking, intrinsic immunity, apoptosis, antigen presentation and microRNA [55–64]. It appears as though a personalized manipulation of the host environment by each parasite is imperative to ensure its survival and reproduction.

1.7 Nutritional basis of intracellular parasitism

Most apicomplexans during their entire intracellular phase are sheltered within a non-fusogenic vacuole, which shields from host defense, but also restricts them from retrieving metabolites (Fig 8). Parasites have therefore invented ingenious strategies; for example, *Toxoplasma*, *Plasmodium* and *Eimeria* remodel their PVM as such to make it permeable to small molecules [65–67]. The PVM of *T. gondii* tachyzoite harbors Gra17 and Gra23 proteins, which enable diffusion of molecules up to 1.3-kDa [65,68]. The PVMs of erythrocytic and hepatic stages of *Plasmodium* also function as molecular sieves allowing the passage of metabolites below 1.4-kDa and 0.85-kDa, respectively [51,66]. *Plasmodium* merozoites also modify the permeability of erythrocyte plasma membrane to obtain a wide range of nutrients from the blood milieu [69,70]. These membrane sieves are very vital for salvaging those nutrients that parasites are unable to produce themselves, as well as for importing the precursors for *de novo* synthesis of complex macromolecules within the parasite.

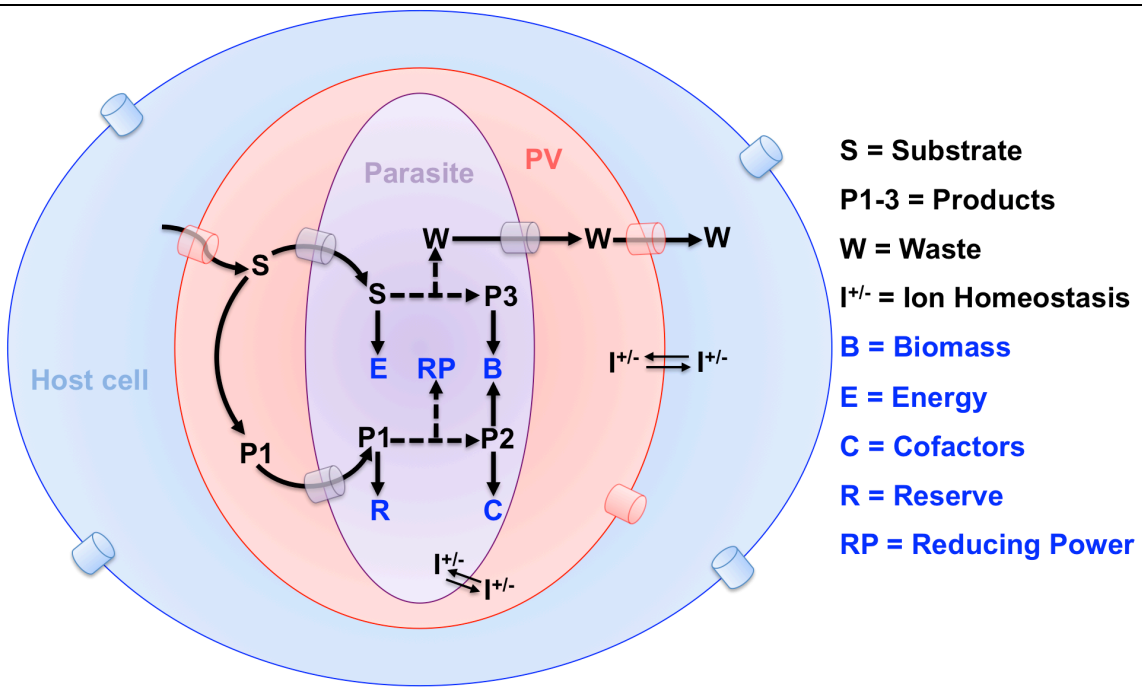

Fig 8: *Abridged depiction of metabolic interactions in a parasite-infected cell.* Most parasites are separated from exogenous nutrients by at least three physical barriers including the plasma membranes of the host cell and parasite, as well as the parasitophorous vacuole membrane. Exemplary flow of metabolic substrates (S) and waste (W) across these membranes and putative transporters are shown. Just as any other cell, parasites require biomass, energy, cofactors, reducing equivalents, ion homeostasis and nutrient stores for reproduction; however, how they fulfill these metabolic necessities differs considerably. Depending on the nutrient, they can either make use of the endogenous pathways and/or salvage from the host cell.

In free-living organisms, the linked pathways of central carbon metabolism (glycolysis, pentose phosphate shunt, TCA cycle) constitute a metabolic hub to ensure the biomass, energy and redox demands during cell proliferation and differentiation [71,72]. While intracellular parasitism has resulted in a net loss of metabolic pathways in apicomplexans, these three core pathways remain conserved in most intracellular parasites [9]. Evenly, parasites have also often preserved synthesis of fatty acids except for few (*e.g.*, *Theileria*), which appear to be auxotrophic [73]. *Toxoplasma* and *Eimeria* genomes also encode pathways to produce most amino acids [74]. By contrast, *Plasmodium* merozoites cannot produce them and satisfy their demands for amino acids largely by degrading erythrocyte-derived hemoglobin in food vacuole [75]. Except for the cytostome-mediated uptake of hemoglobin [76,77], there is no tangible evidence for a typical endocytosis-mediated import in apicomplexans [78]. Hence, once across the PV sieve, metabolites translocate to the parasite *via* substrate-specific transporters located in the plasma membrane. Indeed, these parasites encode a large repertory of transporters (www.membranetransport.org) (Table 1).

Metabolism is also among one of the most affected functional categories in transcriptomics and proteomics studies of host cells infected with *Eimeria*, *Toxoplasma* and *Plasmodium* species, as well as in intracellularly-residing parasites [55,57–59,64,79–81]. These data suggest a robust importance of metabolic interactions between the parasite and host cell. The types of metabolism-associated proteins modulated in parasitized host cells include carbohydrate, lipid and nucleotide pathways, which match across infections. It is also postulated that host mitochondria and ER may donate multiple nutrients to the developing parasites [82], such as lipids, sugar-phosphates, amino acids, fatty acids, glycan intermediates and lipoic acid. Although pending a direct proof, tachyzoites of *Toxoplasma* may also salvage a range of metabolites (metals, sulfate, lipids, amino acids, sugars) by selectively intercepting the host vesicles *via* HOST structures [83] (Fig 7). Not much is known about *Eimeria* species, but given the phylogenetic proximity, their metabolic crosstalk with respective host cells is likely to resemble *T. gondii*, excluding niche-specific variations. *Theileria* and *Babesia* escape the PV immediately after invasion and divide in the cytoplasm of lymphocytes and erythrocytes, respectively. These parasites have more direct access to host-derived nutrients that resonates with their much lower metabolic potential than peers [45].

1.8 Overarching theme and underlying paradigms

Infection, pathogenesis and transmission of apicomplexan parasites depend on distinct abilities of individual pathogens to switch lifecycle stages and exploit host-cell resources including nutrients. By examining how parasites interact with and respond to the nutritional milieus encountered in their host cells, one can learn how to selectively inhibit them. Apicomplexan parasites have much smaller genomes (Table 1), which signifies that they are under different selective pressures when compared to free-living counterparts. In context of this work, our genome-wide comparisons are indicative of a strong evolutionary influence of metabolism in shaping the world of apicomplexan parasites. They have gained or lost selected metabolic pathways, while optimizing their lifecycles with individual host through the long path of co-evolution. *Toxoplasma*, *Plasmodium* and *Eimeria* for example harbor just about 1300-1700 enzymes, which is much less than the typical mammalian

hosts, human and mouse, encoding for approximately 5000 enzymes (Table 1). An equally strong variance with similar pattern is also observed when comparing the number of unique proteins and transporters. In essence, it reflects multiple metabolic dependencies of designated parasites, which may underlie their ultimate adaptation to a strictly intracellular parasitic lifestyle.

Table 1: *Genome-wide comparisons of selected apicomplexan parasites and mammalian hosts*

Organism[a]	**Genome size (Mb)**	**Total genes (Unique proteins)**	**Enzymes (with EC #)**	**Transporters (# with GO term)**
Toxoplasma gondii (ME49)	64	8920 [b]	1700 [b]	304 [b]
Plasmodium falciparum (3D7)	23	5777 [b]	1311 [b]	406 [b]
Eimeria tenella (Houghton)	60	8634 [b]	1556 [b]	216 [b]
Homo sapiens (Human)	3300	23287 (87222) [c]	4974 [d]	2725 [d]
Mus musculus (mouse)	2800	22796 (45557) [c]	4852 [d]	2361 [d]

[a] Only one representative species of each organism is shown.
[b] Deduced from the parasite genome databases, www.ToxoDB.org and www.PlasmoDB.org.
[c] Extracted from The Global Proteome Machine Database (www.thegpm.org).
[d] As annotated in UniProt Database (www.uniprot.org).

Asexual reproduction of parasites within host cells is achieved by consecutive lytic cycles. A single parasite can usually produce 16-64 daughter cells within a nonfusogenic vacuole, which serves as a safe residence for reproduction. Such an efficient replication and concomitant expansion of the PV requires significant nutritional import and synthesis of macromolecules within the parasite. A number of metabolic precursors are likely transported and utilized to produce biomass. However, the mechanisms of import and subsequent metabolic usage of many such nutrients by parasites (metabolic potential) are very much underappreciated. In addition, it is not well understood how the parasite metabolism is rewired and regulated during the lytic cycle and stage differentiation. In specifics, this work aimed to investigate:

- Central carbon metabolism of *Toxoplasma* and *Plasmodium*
- Mechanisms of membrane biogenesis in *Toxoplasma* and *Eimeria*
- Host metabolism as a determinant of the parasite infection (*Eimeria* and *Toxoplasma*)
- Regulation of asexual reproduction in *Toxoplasma*
- Potential anti-parasitic drug targets in the parasite metabolism

The following sections describe and discuss the results generated using *T. gondii*, *P. berghei* and *E. falciformis* as the representative intracellular parasites. Collectively, these parasites have enabled a complementary study on discrete infectious stages of the apicomplexan lifecycle, *e.g.*, tachyzoite, bradyzoite, sporozoite and merozoite (Fig 3). While *T. gondii* has been pivotal in this work owing to relative ease of culture and genetic tractability, *P. berghei* and *E. falciformis* have been imperative to compare and complete the specific findings.

2 RESULTS AND DISCUSSION

2.1 Central carbon metabolism of *Toxoplasma gondii* and *Plasmodium berghei*

Underlying publications/manuscript (for abstracts, please refer to Section 5 on page 53-57):

Host-derived glucose and its transporter in the obligate intracellular pathogen *Toxoplasma gondii* are dispensable by glutaminolysis. Blume M, Contreras DR, Landfear S, Fleige T, Soldati DF, Lucius R, Gupta N; *Proceedings of National Academy of Sciences USA*, 2009, 106(31): 12998-3003 **Appendix A** [84]

A constitutive pan-hexose permease in *Plasmodium* and models for high-throughput screening of anti-malarial sugar analogs. Blume M, Hliscs M, Contreras DR, Sanchez M, Landfear S, Lucius R, Matuschewski K, Gupta N; *FASEB J*, 2011, 25(4): 1218-29 **Appendix B** [85]

Metabolic cooperation of glucose and glutamine is essential for the lytic cycle of obligate intracellular parasite *Toxoplasma gondii*. Nitzsche R, Zagoriy V, Lucius R, Gupta N; *Journal of Biological Chemistry*, 2016, 291(1): 126-41 **Appendix C** [86]

A *Toxoplasma gondii* gluconeogenic enzyme contributes to robust central carbon metabolism and is essential for replication and virulence. Blume M, Nitzsche R, Sternberg U, Gerlic M, Masters SL, Gupta N, McConville MJ; *Cell Host & Microbe*, 2015, 18(2): 210-20 **Appendix D** [87]

A mitochondrial phosphoenolpyruvate carboxykinase ensures glucose-independent survival of the protozoan parasite *Toxoplasma gondii*. Nitzsche R, Tischer M, Zagoriy V, Gupta N **Appendix E** [*submitted*]

Toxoplasma and *Plasmodium* are long known to be voracious consumers of glucose [88,89]. Sugar catabolism through glycolysis does not yield significant CO_2 by feeding of sugar-derived pyruvate in the parasite mitochondrion; instead, it is reduced to lactate, which is excreted in cultures. This results in a lower energy yield (4 moles of ATP per mole of glucose as opposed to 36 moles when completely oxidized in the mitochondria) and loss of 3 carbons as lactate [90]. The phenomenon has been attributed to hypoxic intracellular environment these parasites are regularly faced with, although the quintessence of such an inefficient metabolism is quite intriguing. In other words, the scope and extent to which glucose satisfies the bioenergetic obligations in these parasites has not been adequately understood. We studied how sugar is imported and utilized for biosynthetic purpose, and whether parasites can deploy alternative sources of carbon.

We identified closely related orthologs of facilitative sugar transporter in the genomes of *T. gondii* (*Tg*GT1), *P. berghei* (*Pb*HT1) and *P. falciparum* (*Pf*HT1) (*Appendix A-B*). All three transporters exhibit similar substrate specificities. They can transport glucose, fructose, mannose as well as galactose, albeit with somewhat varying affinities. Surprisingly, genetic ablation of *Tg*GT1 inflicted only a minor 30% defect in the tachyzoite growth, even though sugar import by the parasite was nearly abolished. Virulence of the Δ*tggt1* mutant in mice remained unaffected, indicating dispensability of glucose transport for *in vivo* reproduction of tachyzoites. The ATP-dependent gliding motility and *de novo* synthesis of RNA and proteins in the Δ*tggt1* mutant in standard culture medium were remarkably normal, which implied flexible usage of alternative nutrients. Indeed, we identified glutamine as a second major carbon source used by tachyzoites. Notably, parasites can assimilate

glutamine through the TCA cycle in a constitutive manner regardless of glycolytic flux. The Δ*tggt1* mutant shows induction of glutaminolysis along with activation of gluconeogenesis, which together support the bioenergetic demands in the absence of glucose import (Fig 9) (*Appendix C*).

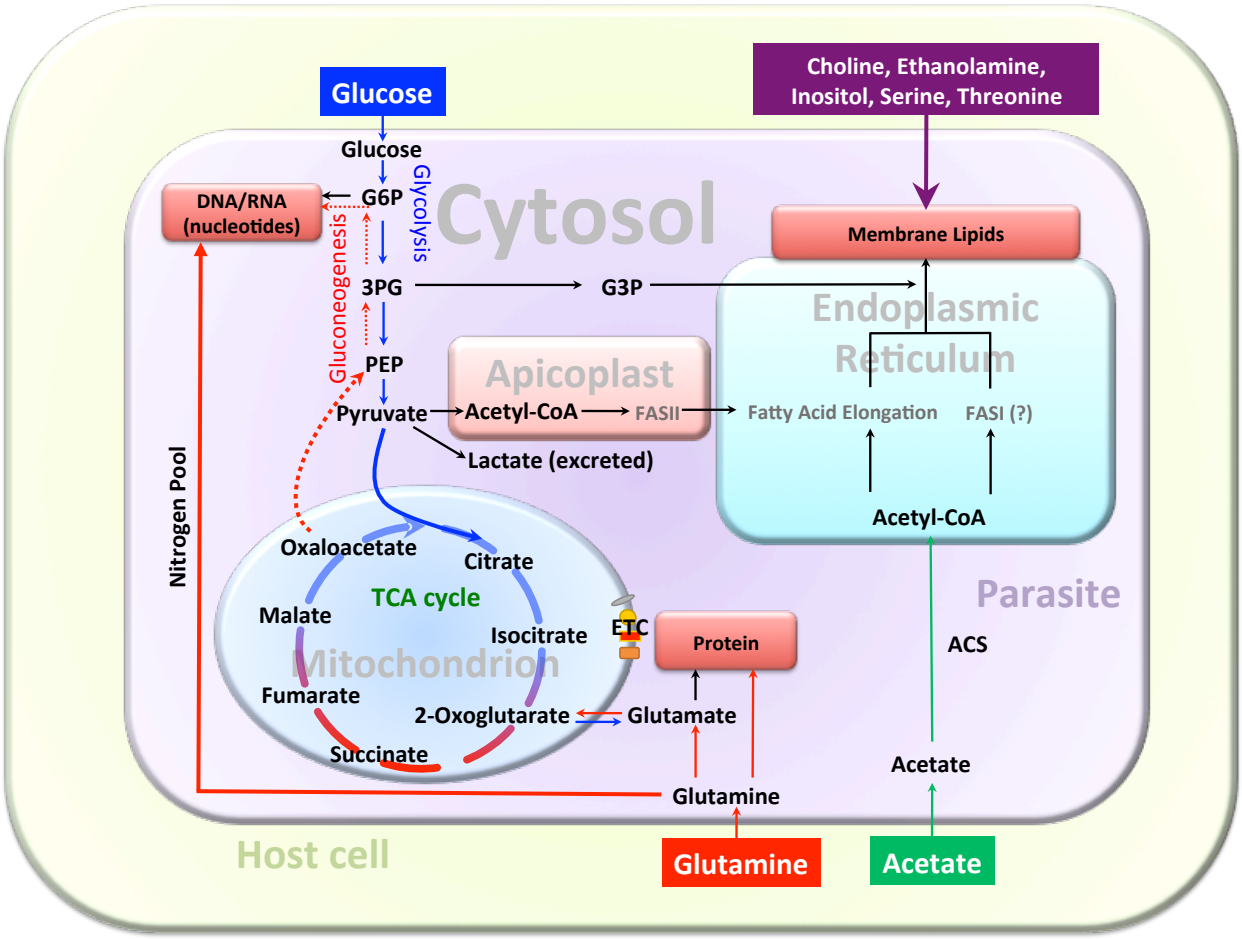

Fig 9: *Central carbon metabolism in the tachyzoite stage of T. gondii*. The model is constructed based on our work, literature and annotations of selected enzymes expressed in the tachyzoite stage (www.ToxoDB.org). Only those metabolites relevant to this work are shown for simplicity. Glucose and glutamine are co-utilized to satisfy the carbon demands for biomass (protein, nucleotides, lipids), energy and reducing equivalents (not depicted). Parasites can also deploy acetate as a carbon source for lipid synthesis when available in culture. Lipid synthesis utilizes acetyl-CoA and 3PG (3-phosphoglycerate), which are mostly derived from glucose. Biogenesis of nucleotides requires ribose 5-phosphate produced by diversion of G6P (glucose-6 phosphate) to the pentose phosphate shunt. Likewise, protein synthesis uses glucose-derived nonessential amino acids, such as serine and glycine (not shown). Glutamine catabolism enables effective biosynthetic utilization of sugar-derived carbon by replenishing the TCA cycle (drained by macromolecule biogenesis in replicating parasites). Glutamine also confers the much-needed nitrogen pool for nucleotides and protein syntheses. Glutamine-derived carbon flux (TCA cycle and gluconeogenesis) can sustain the parasite survival without a severe growth defect, when glycolysis is compromised in tachyzoites. Other nutrients utilized by the parasite for membrane biogenesis include choline, ethanolamine, serine, inositol and threonine. Carbon metabolism is rewired to meet proliferating (intracellular) and non-proliferating (extracellular) demands as well as in response to the available nutrients. ACS, acetyl-CoA synthetase; ETC, electron transport chain; FASI/FASII, fatty acid synthase I or II; G3P, glycerol-3-phosphate; PEP, phosphoenolpyruvate

Extracellular tachyzoites depend on either glucose or glutamine to invade host cells, because the exogenous milieu lacks any substitutive nutrients that can generate adequate energy to facilitate the gliding motility and host-cell invasion. Indeed, a pharmacological inhibition of glutaminolysis or oxidative phosphorylation in the glycolysis-deficient mutant arrests the lytic cycle. Intracellular parasites on the other hand show a much greater resilience, possibly by exploiting intermediates of the host-cell glycolysis and other amino acids. Nonetheless, glucose and glutamine are the two key physiologically important nutrients utilized for the synthesis of macromolecules (ATP, nucleic acid, proteins, lipids). They together furnish a major fraction of total biomass carbon and energy in a co-regulated manner, and either of them is sufficient to support the replication of tachyzoites (*Appendix C*). Ironically, whereas glutamine is capable of driving nucleotide and protein biogenesis when glycolysis is impaired, it falls short in ensuring an optimal fatty acid synthesis, which results in a wide-spectrum deficit of lipids leading to a modest growth defect in the Δ*tggt1* mutant. Lipid synthesis as well as the lytic cycle of the glycolytic mutant can be restored by acetate supplement, which can recompense for the glucose-derived acetyl-CoA for fatty acid synthesis and elongation *via* FASII and FAE pathways in the apicoplast and ER (Fig 9). Such a cooperative metabolism of glucose, glutamine and acetate in tachyzoites resembles the physiology of tumor cells [72,90–92].

Our ensuing work on gluconeogenesis has identified two isoforms of the gluconeogenic enzyme fructose 1,6-bisphosphatase (*Tg*FBP1, *Tg*FBP2) in tachyzoites, which are constitutively expressed (*Appendix D*). It is quite unusual given that expression of gluconeogenic enzymes is repressed when glucose is available to avert a futile cycling of glycolytic metabolites. We found that whereas *Tg*FBP1 is dispensable irrespective of glucose catabolism, *Tg*FBP2 is essential for the parasite survival even when glycolysis is intact. Conditional knockdown of *Tg*FBP2 results in a complete cessation of the parasite growth and virulence in a mouse model. *Tg*FBP2 deficiency translates into altered glycolytic and TCA cycle flux, as well as dysregulation of glycolipid, amylopectin and fatty acid syntheses. We therefore postulate a futile cycle between fructose 1,6-bisphosphatase (gluconeogenic) and phosphofructokinase (glycolytic) enzymes as a novel regulatory mechanism that may allow tachyzoites to rapidly adapt to nutrients available in different host cells. Our work also found two isoforms of phosphoenolpyruvate carboxykinase (PEPCK) protein, only one of which is expressed in the tachyzoite stage ($PEPCK_{mt}$ localized in the mitochondrion; *Appendix E*). Parasites tolerated the deletion of either isoforms, indicating a nonessential role of PEPCK enzymes in normal glucose-replete cultures. They could not survive collective ablations of both $PEPCK_{mt}$ and *Tg*GT1 however, because the mitochondrial isoform is required for glutamine-fueled gluconeogenesis, which in turn sustains the biomass production in proliferating parasites upon glycolytic dysfunction. Once again, these results on $PEPCK_{mt}$-dependent gluconeogenesis converge with cancer cells, which rely on glutamine-derived carbon flux to withstand glucose-independent growth [93].

We also observed that the physiological significance of glucose for *Plasmodium* contrasts with *T. gondii*, even though the former can also assimilate glutamine as a major carbon source [94]. It was not feasible to ablate the *PbHT1* gene without parallel complementation with a functional copy,

which confirms an essential role of glucose for erythrocytic stages of *P. berghei* (*Appendix B*). These results on genetic essentiality of glycolysis have also been independently confirmed by a similar study on *P. falciparum* and *P. berghei* [95]. Our *in silico* searches show that both *Plasmodium* species lack gluconeogenesis, which renders glycolysis essential. Unlike glycolysis, most of the enzymes of TCA cycle have been shown to be nonessential for the asexual growth of *P. falciparum* [96]. These mitochondrial mutants exhibited a normal erythrocytic cycle; however, their development was severely interrupted in the mosquito host. Along the line, we determined that a glucose analog (C3361) potently inhibits the sexual and hepatic development of *P. berghei*. Therefore, it can be concluded that glucose is indispensable for the entire lifecycle of *Plasmodium* species. To pursue a translational application of these findings, we generated transgenic strains of *Saccharomyces cerevisiae* and *P. berghei*, which express hexose transporter of *P. falciparum* in lieu of endogenous permease(s), and as a consequence depend on *Pf*HT1 for their survival. These two models together permit a streamlined *in vitro* prescreening and subsequent *in vivo* assessment of antimalarial sugar analogs. Such a platform should facilitate the early drug development against human malaria.

2.2 Phospholipid biogenesis in tachyzoites of *Toxoplasma gondii*

Intracellular proliferation of tachyzoites and expansion of the PV require substantial synthesis of organelle membranes, which are composed of predominantly neutral and polar lipids. This work explored several primary features of phospholipid metabolism in *T. gondii*. We demonstrate that tachyzoites harbor standard eukaryotic as well as atypical lipids. Phosphatidylcholine (PtdCho) is the most common lipid, followed by phosphatidylethanolamine (PtdEtn), phosphatidylthreonine (PtdThr), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), ethanolamine-phosphorylceramide (EPC), phosphatidylglycerol (PtdGro) and phosphatidate (PtdOH) (Fig 10). Intracellular tachyzoites express a nearly complete set of enzymes, which catalyze the synthesis of major phospholipids using host-derived carbon sources, namely glucose, glutamine and acetate.

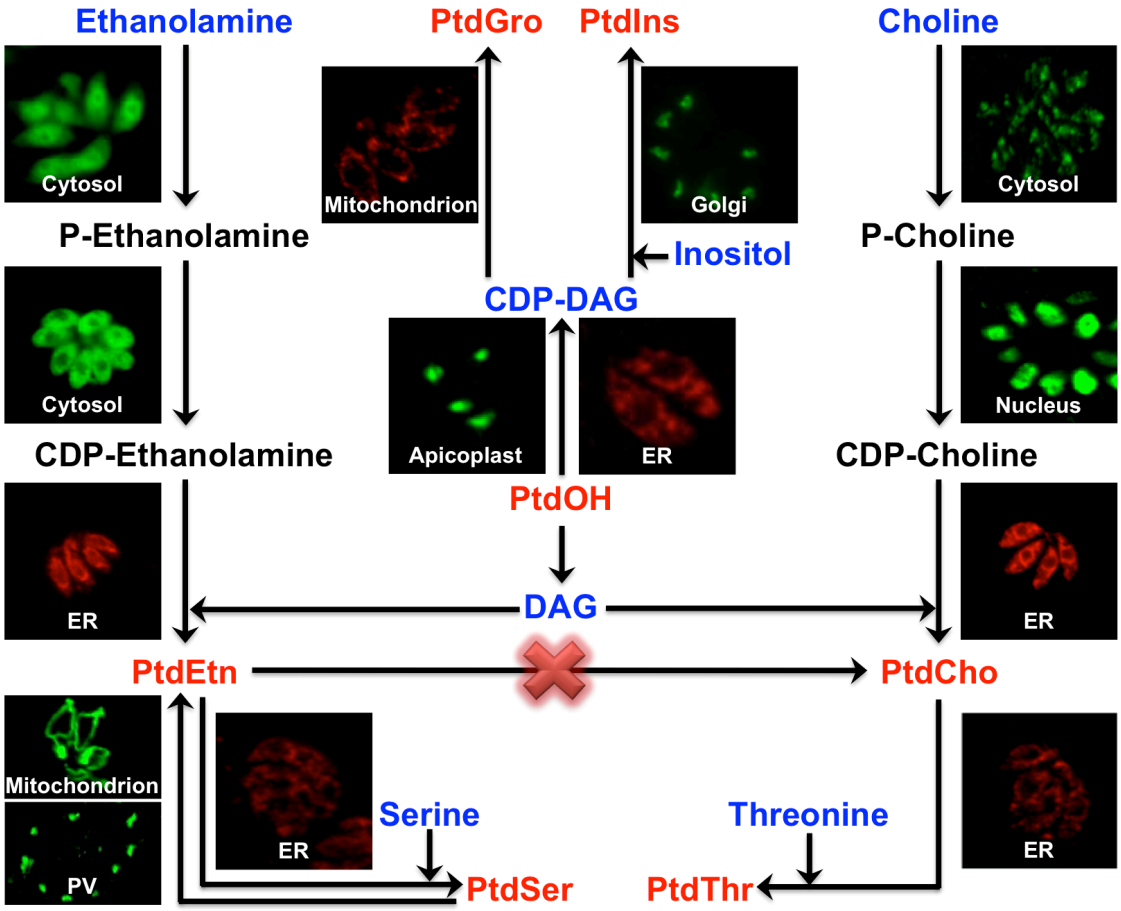

Fig 10: *Pathways of phospholipid syntheses in the tachyzoite stages of T. gondii*. The model is constructed primarily based on our results. Lipid precursors are shown in blue; the intermediates of lipid synthesis are in black; and phospholipids are in red. Phospholipid synthesis begins with the synthesis of precursor lipid (PtdOH), which is converted to diacylglycerol (DAG) for making PtdCho and PtdEtn in the ER. PtdEtn can also be generated in the mitochondrion and PV by decarboxylation of PtdSer. Unlike mammalian cells, PtdEtn is not methylated to make PtdCho (red cross). PtdThr and PtdSer are produced exclusively in the parasite ER. PtdOH in the ER and apicoplast also yields CDP-DAG, which serves as a substrate for the synthesis of PtdIns in the Golgi bodies and PtdGro in the mitochondrion. Glycerol backbone and fatty acids are originally derived from glycolysis (Fig 9). Phospholipid synthesis is significantly induced or repressed when tachyzoites switch between intracellular and extracellular phases, respectively, during the lytic cycle.

Synthesis of phospholipids is intimately interconnected, which occurs mainly in the endoplasmic reticulum, mitochondrion, Golgi bodies, apicoplast and PV (Fig 10). Our studies demonstrate the existence of the CDP-choline and CDP-ethanolamine pathways to generate PtdCho and PtdEtn, respectively. Their syntheses involve PtdOH-derived DAG and CDP-conjugated intermediates of choline or ethanolamine. Tachyzoites can also decarboxylate PtdSer to synthesize PtdEtn. In converse, PtdSer is made from PtdEtn by substituting the head groups (ethanolamine by serine). A similar base-exchange pathway exists for producing PtdThr, possibly utilizing PtdCho as a donor lipid (substitution of choline by threonine). Unlike others, synthesis of PtdIns and PtdGro utilizes CDP-DAG as a precursor instead of DAG. Highly compartmentalized syntheses of most lipid precursors suggest substantial inter-organelle trafficking. Following text describes biogenesis and biological relevance of major phospholipids during the lytic cycle of tachyzoites.

2.2.1 Phosphatidylcholine synthesis – an anti-parasitic drug target

Underlying publications (for abstracts, please refer to Section 5 on page 58-59):

Selective disruption of phosphatidylcholine metabolism of the intracellular parasite *Toxoplasma gondii* arrests its growth. Gupta N, Zahn MM, Coppens I, Joiner KA, Voelker DR; *Journal of Biological Chemistry*, 2005, 280(16): 16345–53 **Appendix F** [97]

Conditional mutagenesis of a novel choline kinase demonstrates the plasticity of phosphatidylcholine biogenesis and gene expression in *Toxoplasma gondii*. Sampels V, Hartmann A, Dietrich I, Coppens I, Sheiner L, Striepen B, Herrmann A, Lucius R, Gupta N; *Journal of Biological Chemistry*, 2012, 287(20): 16289-99 **Appendix G** [98]

PtdCho is the most abundant lipid in the tachyzoite membranes. It amounts up to 75% of total phospholipids (*Appendix F*). Our results provide important insights into PtdCho synthesis of *T. gondii*. Similar to mammalian cells [99], tachyzoites express the three-step CDP-choline pathway to synthesize PtdCho using choline and DAG (Fig 11). They however lack the enzyme activity for PtdEtn methyltransferase and therefore unable to make PtdCho by methylating PtdEtn (Fig 10). Unlike *Plasmodium* [100,101], tachyzoites are also unable to produce PtdCho using serine and ethanolamine as precursors because they do not harbor the plant-type serine decarboxylase and phosphoethanolamine methyltransferase enzymes, respectively. Interception of host's low-density lipoproteins containing PtdCho offers an alternative route to acquire PtdCho. Our assays using LDL particles loaded with a fluorescent lipid probe did not reveal a marked import of PtdCho by intracellular tachyzoites [102]. Likewise, metabolic labeling of host-free parasites with fluorescent PtdCho did not show any noteworthy uptake. As a consequence, tachyzoites depend on their *de novo* CDP-choline pathway to produce majority of PtdCho.

The dependence of *T. gondii* on choline for PtdCho synthesis renders its CDP-choline pathway vulnerable to pharmacological inhibition by choline analogs. We assessed dimethylethanolamine (DME), which was innocuous to human host cells and thus inhibited the lytic cycle of tachyzoites

in a rather selective manner (*Appendix F*). The host-cell lysis was greatly reduced and the parasite yield was declined by almost 3 orders of magnitude when treated with DME. Parasites cultured with permissible amounts of DME accumulated high level of phosphatidyldimethylethanolamine (PDME, a PtdCho analog), which was inversely correlated with the content of PtdCho. Electron microscopy revealed that DME dramatically altered the membrane organization in developing parasites. The newly forming progeny were most profoundly affected, because they seem unable to obtain sufficient PtdCho from host cells, which could thwart the DME-mediated inhibition of *de novo* PtdCho synthesis (Fig 11). Anti-parasitic effect of DME appears to be a result of accrued PDME that is not methylated to PtdCho due to lack of a PtdEtn methyltransferase in *T. gondii*.

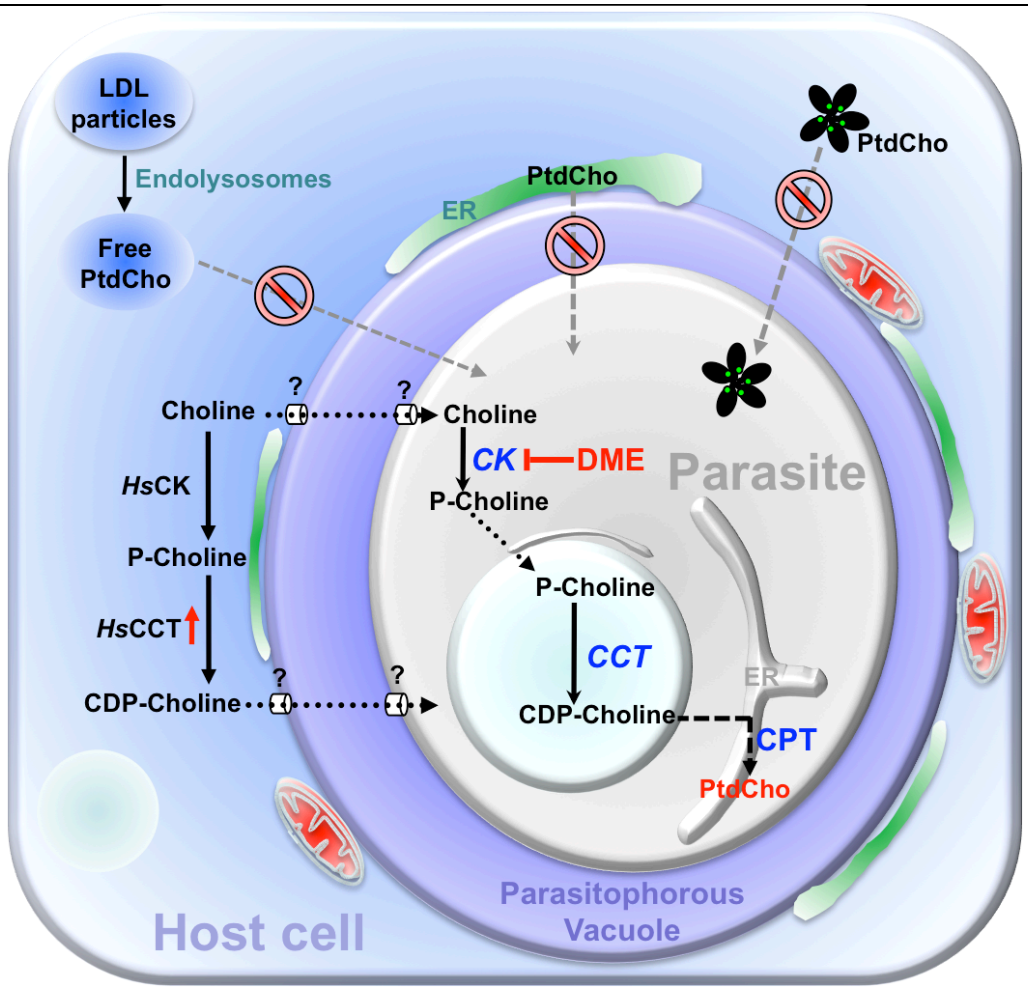

Fig 11: *Current model of PtdCho biogenesis in T. gondii tachyzoites*. The parasite seems to be a choline auxotroph for making PtdCho *via* the three-step CDP-choline pathway localized in three different organelles. The first enzyme CK is cytosolic, the second and rate-limiting enzyme CCT is nuclear, and the last one (CPT) catalyzing the synthesis of PtdCho using CDP-choline and DAG is located in the ER. Unlike *Plasmodium*, scavenging of host PtdCho (LDL-derived or *via* host ER-PVM association) likely does not contribute to PtdCho biogenesis. Host CCT (*Hs*CCT) is upregulated in infected human fibroblasts, but its functional relevance is unclear. Dotted arrows denote transport processes. Image is redrawn from the PhD thesis of Vera Sampels [102]. CK, choline kinase; CCT, CTP-phosphocholine cytidylyltransferase; CPT, choline phosphotransferase; ER, endoplasmic reticulum; LDL, low-density lipoprotein

In follow-up work, we showed that the synthesis of PtdCho is initiated by a novel choline kinase (*Tg*CK), which forms oligomers in the tachyzoite cytosol (*Appendix G*). The corresponding gene could not be directly deleted, indicating an essential nature of choline kinase activity. Conditional mutagenesis of the *Tg*CK gene by promoter displacement in tachyzoites attenuated the protein level. The activity could not be eliminated however, which led to the unexpected identification of two additional shorter isoforms encoded by a cryptic promoter. We also observed a quantitative concordance between the enzyme activity and PtdCho synthesis, which entails a dependence of parasites on *de novo* CDP-choline route. The conditional mutant displayed a surprisingly normal *off-state* growth despite a 35% decline in total PtdCho content. The data reveal a compositional flexibility in the tachyzoite membranes while advocating against the salvage of host PtdCho in sufficient amounts to hedge a knockdown of the CDP-choline pathway. Meanwhile, we have also generated a conditional mutant of the second enzyme (CCT), which exhibits 50% impairment in growth when its expression is turned off [102]. Future work involves creating and phenotyping the mutants of CPT, the last enzyme of PtdCho synthesis located in the ER (Fig 11).

2.2.2 Plasticity of phosphatidylethanolamine biogenesis

Underlying publications (for abstracts, please refer to Section 5 on page 60-61):

The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase. Gupta N, Hartmann A, Lucius R, Voelker DR; *Journal of Biological Chemistry*, 2012, 287(27): 22938-47 **Appendix H** [103]

Phosphatidylethanolamine synthesis in the parasite mitochondrion is required for efficient growth but dispensable for survival of *Toxoplasma gondii*. Hartmann A, Hellmund M, Lucius R, Voelker DR, Gupta N; *Journal of Biological Chemistry*, 2014, 289(10): 6809-24 **Appendix I** [104]

PtdEtn is the second most abundant phospholipid in *T. gondii* tachyzoites, accounting for 15-20% of total phospholipids. Parasites can synthesize PtdEtn in the mitochondrion and parasitophorous vacuole by PtdSer decarboxylation as well as in the ER through the CDP-ethanolamine pathway (*Appendix H-I*). Two different PtdSer decarboxylase enzymes, *Tg*PSD1$_{mt}$ and *Tg*PSD1$_{pv}$ localized in the mitochondrion and PV respectively, are expressed in tachyzoites (Fig 12). Genetic ablation of *Tg*PSD1$_{mt}$ expression caused ≈45% impairment in the mutant's growth. Unexpectedly, no obvious change in the total PtdEtn content of the mutant was observed, signifying the activation of compensatory routes. Indeed, metabolic labeling with ethanolamine revealed induction of the CDP-ethanolamine pathway in the ER when *Tg*PSD1$_{mt}$ was repressed. Consistently, depletion of ethanolamine intensified the growth defect in the *Tg*PSD1$_{mt}$ mutant, which could be partially rescued by add-back assay. Hence, both routes are not mutually exclusive and specific exchange of lipid seems to exist between the mitochondrion and ER of tachyzoites. Such a phenomenon has already been reported to occur in mammalian cells [99,105]. The presence of lipid trafficking and importance of the CDP-ethanolamine pathway are yet to be established in *T. gondii*.

Our work also revealed that tachyzoites secrete a soluble form of PtdSer decarboxylase to the PV *via* dense granules. Unlike other PSDs, which require detergent-solubilized substrate, *Tg*PSD1$_{pv}$ can decarboxylate even the liposomal PtdSer with a high affinity (K_m, 67 μM). It is a novel protein because a secreted, soluble and interfacially active PSD has not been previously described from any organism. Remarkably, tachyzoites can survive genetic deletion of *Tg*PSD1$_{pv}$ with no evident defect in growth and lipid composition, which is probably due to acquisition of PtdEtn from host cells [106] (Fig 12). In this regard, a close association of the PVM with the host mitochondria and ER might serve as the privileged sites of lipid exchange. Our initial work found that the parasite genome encodes at least four P4-ATPase-type permeases, which may facilitate transport of host-derived lipids to the parasite body. Such a resourceful PtdEtn synthesis bestows a considerable metabolic plasticity to the tachyzoite stage of *T. gondii* (Fig 12).

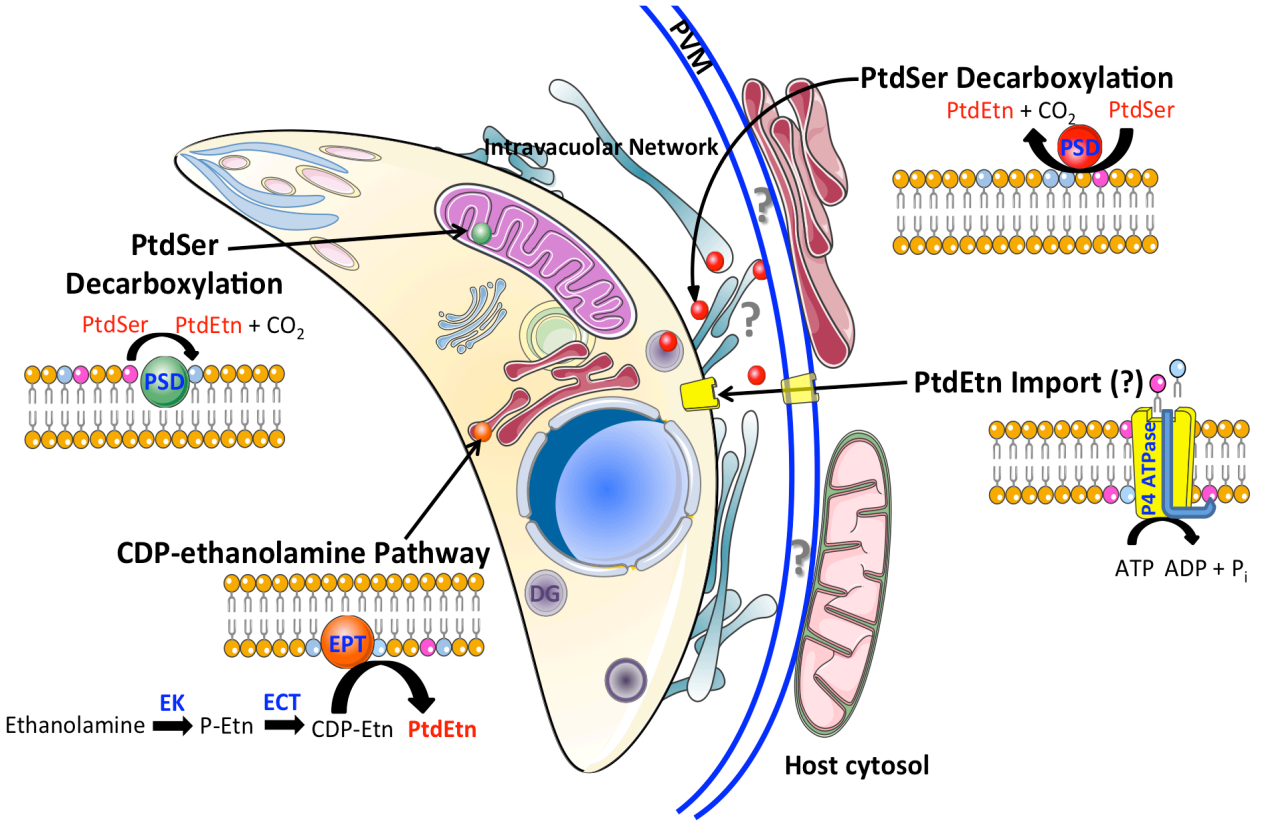

Fig 12: *Multiple routes of PtdEtn biogenesis in T. gondii tachyzoites.* Three distinct pathways operate for making PtdEtn in different organelles. The mitochondrial and secretory variants of PtdSer decarboxylase (PSD) generate PtdEtn by decarboxylating PtdSer in the mitochondrion and PV (and maybe in dense granules). PtdEtn synthesis *via* the CDP-ethanolamine pathway occurs in the ER. The parasite may also acquire PtdEtn from the host ER and/or mitochondria (associated with PVM). Tachyzoites also encode at least 4 putative lipids transporters (P4-ATPase type), whose locations, functions and relevance are not known. In short, multiple sites of PtdEtn biogenesis collaborate to realize a robust membrane biogenesis, and thereby ensure the parasite survival upon nutritional perturbations. Image is adapted from the PhD dissertation of Anne Hartmann [106]. DG, dense granule; EK, ethanolamine kinase; ECT, CTP-phosphoethanolamine cytidylyltransferase; EPT, ethanolamine phosphotransferase

2.2.3 Functional speciation of phosphatidylserine and phosphatidylthreonine

Underlying publications (for abstracts, please refer to Section 5 on page 62-63):

Phosphatidylthreonine and lipid-mediated control parasite virulence. Arroyo-Olarte RD, Brouwers JF, Kuchipudi A, Helms JB, Biswas A, Dunay IR, Lucius R, Gupta N; *PLoS Biology,* 2015, 13(11): e1002288 **Appendix J** [107]

Optogenetic monitoring identifies phosphatidylthreonine-regulated calcium homeostasis in *Toxoplasma gondii*. Kuchipudi A, Arroyo-Olarte RD, Hoffmann F, Brinkmann V, Gupta N; *Microbial Cell*, 2016, 3(5): 215-23 **Appendix K** [108]

Phosphatidylthreonine: An exclusive phospholipid regulating calcium homeostasis and virulence in a parasitic protist. Arroyo-Olarte RD and Gupta N; *Microbial Cell*, 2016, 3(5): 189-190 (micro-review) **Appendix L** [109]

Most organisms across the tree of life use a limited repertoire of phospholipids [105]. This study demonstrates the natural expression and genetic origin of an exclusive lipid PtdThr that is in fact the third most abundant phospholipid in tachyzoites (5-10%) (*Appendix J*). PtdThr is related to PtdSer, a near-universal lipid present only as a minor phospholipid in the tachyzoite membranes (2-3%). Our work identified two closely related enzymes *Tg*PTS (PtdThr synthase) and *Tg*PSS (PtdSer synthase), located in the parasite ER, where they perform the synthesis of PtdThr and PtdSer, respectively. Both enzymes utilize PtdEtn and/or PtdCho as the donor lipids to catalyze the base-exchange reaction (substitution of ethanolamine in PtdEtn or of choline in PtdCho by serine or threonine). Unlike *Tg*PSS, orthologs of *Tg*PTS could only be found in selected parasitic (*Neospora*, *Eimeria*, *Phytophtora*) and free-living (*Perkinsus*) chromalveolates. The *Tg*PSS gene could not be deleted in tachyzoites, indicating an essential role of PtdSer for the lytic cycle. Parasites did survive disruption of the *Tg*PTS gene, though the mutant showed severely compromised lytic cycle and virulence (Fig 13). Surprisingly, the Δ*tgpts* strain did not exhibit impaired replication; instead, the phenotype was caused by a reduced gliding motility, which blighted the downstream events of parasite egress and host-cell invasion. The PTS mutant can prevent toxoplasmosis in a mouse model, which endorses its potential clinical utility as a metabolically attenuated *vaccine*.

The PTS mutant lacking PtdThr showed a proportionate increment in PtdSer, which is reversed by genetic complementation (*Appendix J*). In line, we observed an apparent increase in the level of another anionic lipid, PtdIns; however only when PtdSer content was restored to a normal level in a conditional double mutant of *Tg*PSS and *Tg*PTS. Such a reversible and proportionate balancing of the three anionic lipids in tachyzoites appears to maintain the parasite replication, but certainly unable to uphold the virulence of the Δ*tgpts* mutant. It is therefore plausible that the parasite has evolved PtdThr to optimize its lytic cycle, while satisfying the customary roles of anionic phospholipids in membrane biogenesis. In conjunction with aforesaid text (*Section* 2.2.1 and 2.2.2), these data also reinforce a compositional flexibility in the parasite membranes, which may be pivotal to the widespread survival of *T. gondii*.

In succeeding work, using our newly adapted method based on a gene-encoded calcium indicator GCaMP6s, we showed that the loss of PtdThr depletes calcium stores in intracellular tachyzoites, which leads to dysregulation of cytosolic calcium and accordingly impairs the gliding motility and egress (*Appendix K-L*). Consistently, the parasite motility as well as egress can be entirely restored by ionophore-induced mobilization of calcium in the mutant. Collectively, these results suggest a novel regulatory function of PtdThr in calcium signaling of a prevalent parasitic protist, as well as indicate adaptive and functional speciation of PtdThr from an otherwise-conserved lipid PtdSer. Notably, the parasite also harbors a plant-like pathway to make threonine, an amino acid that is essential for mammalian cells. It therefore appears as though serine-threonine homeostasis in *T. gondii* is quite distinct from its host. Our work has already identified 4 out of 5 enzymes of *de novo* threonine synthesis, which provide a strong basis for future research on the mechanism, evolution and therapeutic potential of threonine/PtdThr syntheses pathways.

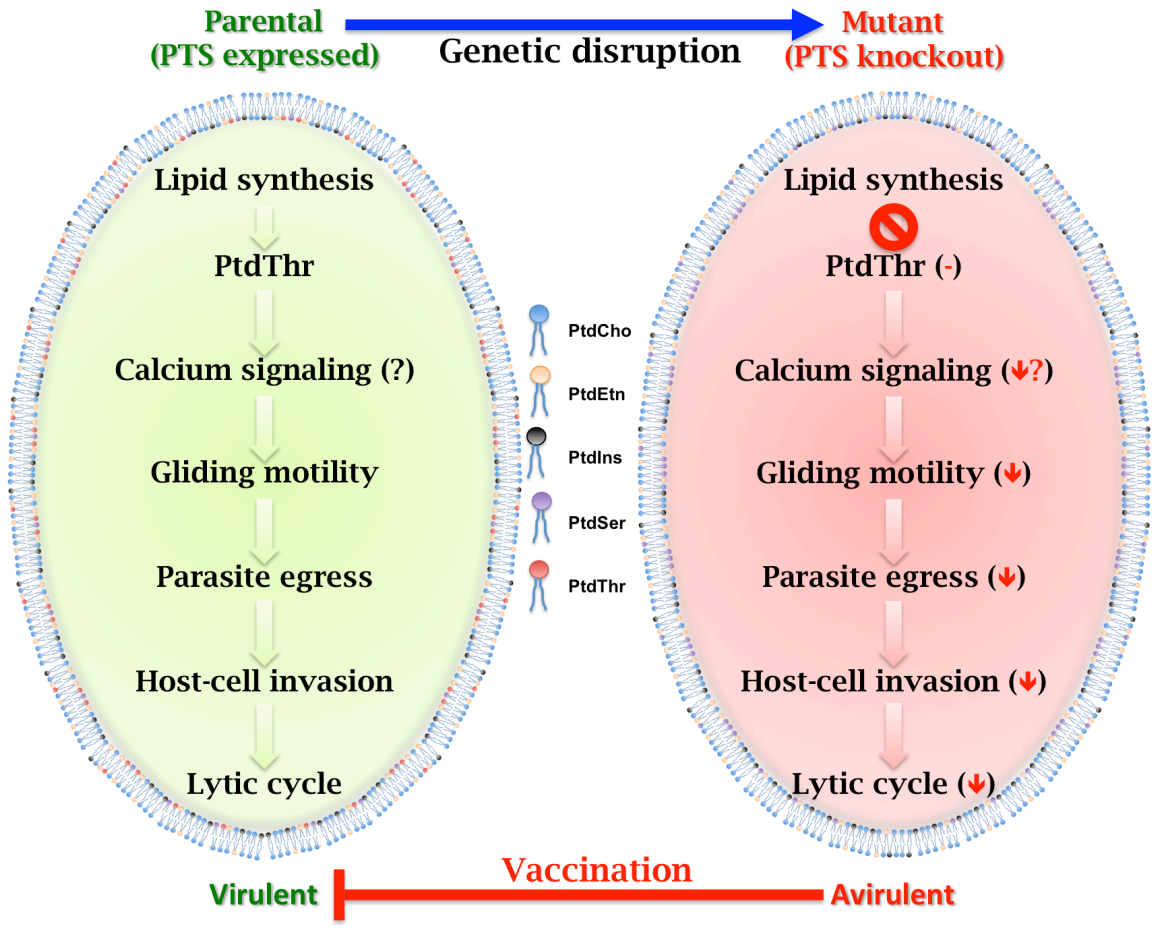

Fig 13: *PtdThr-mediated control of lytic cycle and virulence in T. gondii*. The parental tachyzoites express a PtdThr synthase (PTS). A disruption of the PTS gene results in parasites that are unable to make PtdThr, which compromises the sequential events of calcium discharge in the parasite cytosol, gliding motility, egress from parasitized cells and entry into new host cells. As a consequence, the PTS mutant displays a severely impaired lytic cycle in human cells and attenuated virulence in a mouse model. The mutant can confer potent immunity to surviving animals against the hypervirulent as well as cyst-forming parasite strains.

2.2.4 Biogenesis of phosphatidylinositol and phosphatidylglycerol

Underlying manuscript (for abstract, please refer to Section 5 on page 64):

Two distinct CDP-diacylglycerol synthases in the parasite ER and apicoplast cooperate to ensure lipid biogenesis in *Toxoplasma gondii*. Kong P, Ufermann CM, Yin Q, Suo X, Helms JB, Brouwers JF, Gupta N
Appendix M [*submitted*]

Phospholipid synthesis begins with the conversion of PtdOH into precursor lipids, DAG or CDP-DAG. The latter serves as a precursor for the synthesis of PtdCho and PtdEtn, while CDP-DAG is utilized to synthesize PtdIns and PtdGro (Fig 10). We identified two phylogenetically divergent CDP-DAG synthases in tachyzoites (*Appendix M*), of which the eukaryotic-type *Tg*CDS1 is located in the ER, and prokaryotic-type *Tg*CDS2 in the apicoplast. Conditional mutagenesis of *Tg*CDS1 severely attenuated the parasite growth, which was abolished by subsequent deletion of *Tg*CDS2. The conditional mutant of *Tg*CDS1 was dramatically compromised in its virulence in the mouse. Moreover, animals surviving infection with the mutant became immunized to challenge infection with the hypervirulent strain of *T. gondii*. The mutants revealed significantly reduced PtdIns level upon chemical repression of *Tg*CDS1, even though total phospholipids were markedly increased. On the other hand, the ablation of *Tg*CDS2 (but not of *Tg*CDS1) led to an evident decrease in PtdGro without perturbing PtdIns. In other words, the two enzymes are required for producing different lipids, namely PtdIns and PtdGro, indicating their complementary roles in membrane biogenesis. Our work also suggests that the host is unable to meet the requirement of CDP-DAG when its synthesis within the parasite is compromised. Physiological essentiality and phylogenetic divergence of CDP-DAG synthesis route offer an opportunity to selectively inhibit the parasite.

Our succeeding work has revealed the autonomous syntheses of PtdIns and PtdGro in *T. gondii*, which occurs in Golgi bodies and mitochondrion, respectively. Taken together, the data show a '*division of labor*' model of lipid biogenesis, in which two separate pools of CDP-DAG originating in the ER and apicoplast are utilized to synthesize PtdIns in the Golgi bodies and PtdGro in the mitochondrion (Fig 14). A knockout mutant of one of the underlying enzymes, PtdIns synthase (PIS), could not be generated, signifying its essential nature. Making of a workable conditional mutant was also not feasible, as the transgenic parasites did not allow an adequate repression of the enzyme activity. Eventually, we were successful in destabilizing PIS transcript by Cre-LoxP-mediated excision of 3'UTR, which considerably attenuated the tachyzoite growth in nascent cultures. However the mutant strains adapted rapidly following few passages, which prevented biochemical phenotyping of the mutant. Nonetheless, the results do indicate that parasites are not competent in scavenging host-derived PtdIns in sufficient amounts to bypass a disruption of its endogenous lipid synthesis. We are currently using alternative approaches (dominant negative, CRISPR/Cas9) to obtain a stable mutant to assess the functional importance of PtdIns. Similar work is also being performed on the two enzymes of PtdGro synthesis (Fig 14).

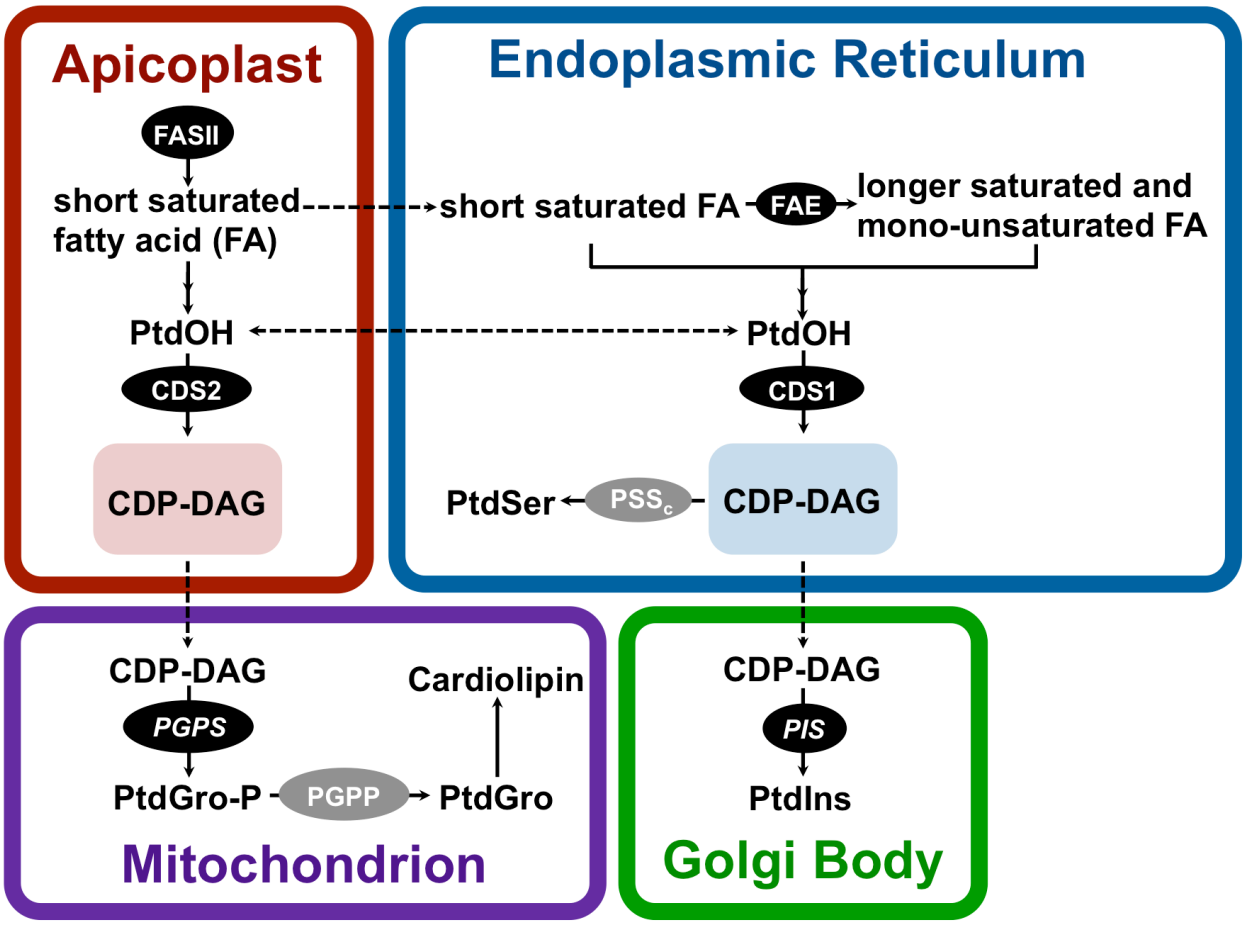

Fig 14: *Synthesis of PtdIns and PtdGro in the tachyzoite stage of T. gondii*. PtdOH serves as a precursor for CDP-DAG, providing the phospholipid backbone. Catalysis of PtdOH into CDP-DAG is executed by *Tg*CDS1 in the ER and by *Tg*CDS2 in the apicoplast. *Tg*CDS1-derived CDP-DAG is used to make PtdIns in the Golgi body (and maybe PtdSer in the ER), while CDP-DAG originating from *Tg*CDS2 facilitates PtdGro and cardiolipin syntheses in the mitochondrion. FASII performs *de novo* synthesis of fatty acids (apicoplast) that are elongated in the ER. Identities of the gray-shaded enzymes (CDP-DAG-dependent PSS_c, PGPP) are not known. Dotted arrows indicate inter-organelle transport of lipids. PtdIns is likely transported from Golgi to the ER for the synthesis of GPI anchors (not shown). CDS, CDP-DAG synthase; FASII, fatty acid synthase II; FAE, fatty acid elongase; PGPP, PtdGro-phosphate phosphatase; PGPS, PtdGro-phosphate synthase; PIS, PtdIns synthase; PSS_c, PtdSer synthase (CDP-DAG-dependent type)

2.3 Phospholipid syntheses in sporozoites of *Eimeria falciformis*

Underlying manuscript (for abstract, please refer to Section 5 on page 65):

Expression of exclusive lipids and autonomous membrane biogenesis indicate a host-independent lifestyle of *Eimeria* sporozoites. Kong P, Brandt S, Brouwers JF, Helms JB, Lucius R, Gupta N

Appendix N [*in prep.*]

The study of sporozoite stage metabolism is profoundly interesting because this is the only freely developing stage of apicomplexan parasites. It allows assessment of the 'true' metabolic potential of a parasite (independent of host cell), as well as offers alternative targets to block transmission. While *Toxoplasma* and *Plasmodium* species do enable a comprehensive dissection of apicomplexan biology, examining the sporozoite stage metabolism is practically challenging because oocysts are produced in a feline or mosquito host, respectively. Hence, we implemented *E. falciformis*, which completes its entire lifecycle in the rodent host and grants a decent model to study the sporozoite stage. Even though our sustained efforts to stably manipulate the *E. falciformis* genome have been futile, we have achieved a feasible transient expression system in sporozoites. In conjunction with the tachyzoite stage of *T. gondii* (a surrogate model), we have started exploring carbon metabolism and membrane biogenesis in *Eimeria* sporozoites. The following results show our advanced-stage work on phospholipid syntheses in the sporozoite stage of *E. falciformis* (*Appendix N*).

In-depth lipidomics analyses of sporozoites purified from the sporulated oocysts revealed that the membrane composition of *Eimeria* sporozoites is similar to that of *T. gondii* tachyzoites excluding few exceptions. PtdCho (79%) and PtdEtn (13%) are the two most abundant lipids accounting for more than 90% of total phospholipids in *Eimeria*. Sporozoites also express PtdThr (6%), a rare lipid shared by tachyzoites, but absent in most organisms including the mouse host. Interestingly, PtdIns in sporozoites is not as abundant as in tachyzoites (1.5% *vs.* 5%), and PtdSer makes up only a rather minor (0.5%) lipid fraction. In contrast to tachyzoites, which express ethanolamine-phosphorylceramide instead of sphingomyelin, sporozoites harbor inositol-phosphorylceramide (IPC). There is no evidence for the natural expression of EPC and IPC in mammalian cells, but they have been reported to occur in kinetoplastid parasites [110].

Most of the core enzymes of lipid synthesis are expressed in the sporozoite stage of *E. falciformis*, indicating its autonomous membrane biogenesis (Fig 15). Our immunofluorescence localization studies in *T. gondii* tachyzoites and *E. falciformis* sporozoites show that the latter stage harbors two distinct CDP-DAG synthases in the ER and apicoplast, and a PtdIns synthase to make PtdIns in the Golgi bodies. In addition, they express the CDP-choline and CDP-ethanolamine pathways in the ER to synthesize PtdCho and PtdEtn respectively, and a second mitochondrial route to make PtdEtn by decarboxylation of PtdSer. Two discrete PSD proteins are expressed in the parasite mitochondrion and dense granules (secreted into the PV after invasion of host cells). As observed in tachyzoites, the base-exchange type PtdSer and PtdThr synthases are present in the ER, while

PtdGro and cardiolipin are generated in the parasite mitochondrion. Unlike *Plasmodium* but akin to *T. gondii*, *Eimeria* lacks the serine decarboxylase and phosphoethanolamine N-methyltransferase pathway; hence sporozoites cannot make PtdCho using serine or ethanolamine as a precursor.

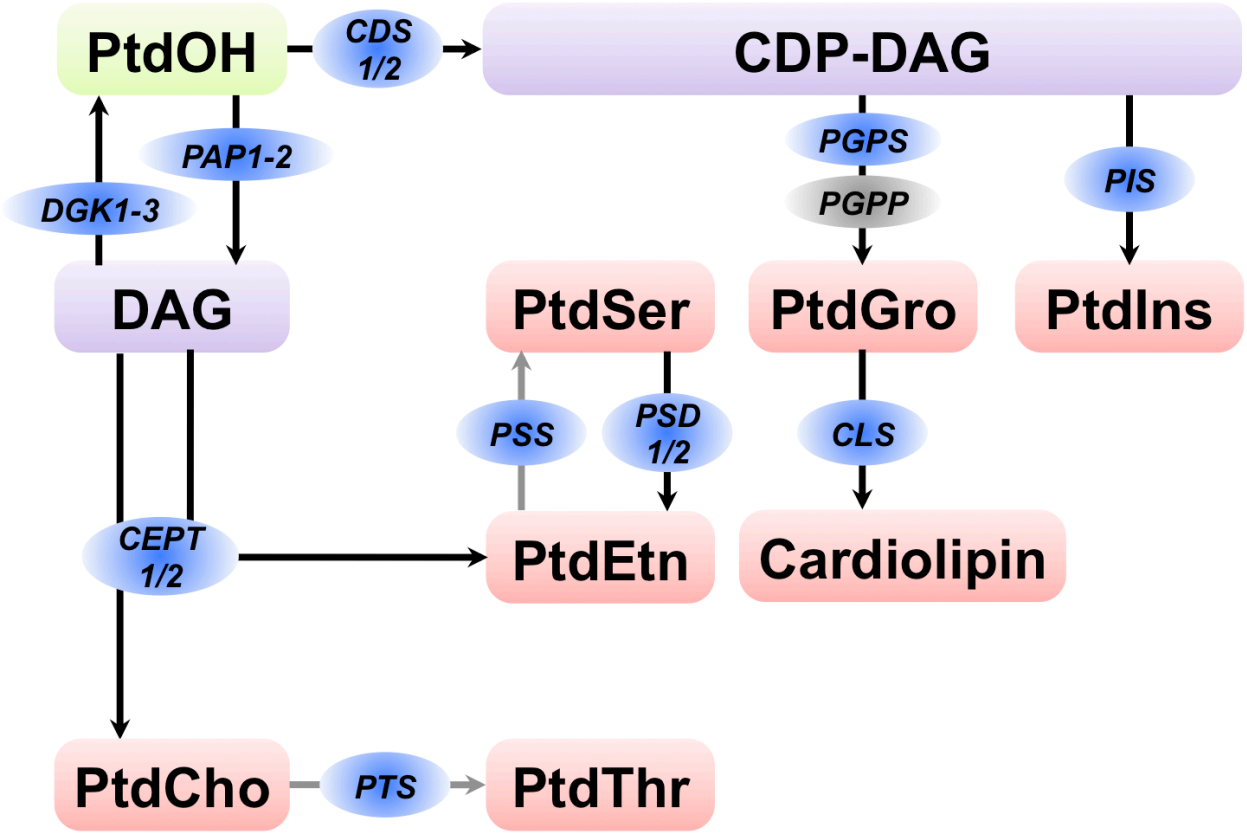

Fig 15: *Syntheses of phospholipids in the sporozoite stage of E. falciformis*. PtdCho, PtdEtn, PtdThr and PtdIns are the major phospholipids present in sporozoites. PtdSer, PtdGro and PtdOH account for minor fractions. Synthesis of most lipids is inter-linked and appears to be somewhat redundant due to multiple isoforms of a given enzyme (see numbers in image) or due to more than one ways of synthesis (*e.g.*, PtdEtn). The gray arrows denote an unclear mode of base-exchange reaction; for instance, PtdSer and PtdThr may be made in a converse manner from PtdCho and PtdEtn, respectively. Identity of PGPP (gray oval) is not clear. CDS, CDP-DAG synthase; CLS, cardiolipin synthase; CEPT, choline-ethanolamine phosphotransferase; DGK, diacylglycerol kinase; PAP, PtdOH phosphatase; PGPP, PtdGro-phosphate phosphatase; PGPS, PtdGro-phosphate synthase; PIS, PtdIns synthase; PSD, PtdSer decarboxylase; PSS, PtdSer synthase; PTS, PtdThr synthase

We were able to confirm the functions of *Ef*PIS and *Ef*PTS, so far. *Ef*PIS was overexpressed in *E. coli*, which lacks the natural synthesis of PtdIns. Recombinant bacteria expressing *Ef*PIS exhibited inositol-dependent synthesis of PtdIns. Equally, *Ef*PTS could rescue the lytic cycle of a tachyzoite mutant with disrupted PtdThr synthesis. Even though many enzymes are still to be functionally characterized, our groundwork on lipid biogenesis and gene manipulation of sporozoites provides a sound basis to elucidate the physiological importance of lipid synthesis in *E. falciformis*, *in vivo*.

2.4 Host determinants of coccidian development

Underlying publications/manuscript (for abstracts, please refer to Section 5 on page 66-68):

Eimeria falciformis infection of the mouse caecum identifies opposing roles of IFNγ-regulated host pathways for the parasite development. Schmid M, Heitlinger E, Spork S, Mollenkopf HP, Lucius R, Gupta N; *Mucosal Immunology*, 2014, 7(4): 969-82 **Appendix O** [111]

Apicomplexan parasite, *Eimeria falciformis*, co-opts host tryptophan catabolism for life cycle progression in the mouse. Schmid M, Lehmann MJ, Lucius R, Gupta N; *Journal of Biological Chemistry*, 2012, 287(24): 20197-207 **Appendix P** [112]

Apicomplexan parasites, *Toxoplasma gondii* and *Eimeria falciformis*, induce and co-opt a master transcription factor cFos in mammalian host cell. Ren B, Schmid M, Mollenkopf HP, Heitlinger E, Gupta N **Appendix Q** [*in prep.*]

Previous sections describe metabolic potential of selected apicomplexan parasites and its impact on the asexual development, pathogenesis and adaptation. To ascertain the design principles of intracellular parasitism, it is equally imperative to investigate how these parasites exploit host cells for their survival and reproduction. At the outset, we employed *E. falciformis* infection of mouse to deduce the modulation of host transcriptome using microarray technology (*Appendix O*). Our gene expression analyses of the parasitized caeca samples (enriched in epithelial cells) identified a retinue of host proteins modulated by the parasite. Most prominent mouse genes induced during the onset of asexual and sexual development of *Eimeria* comprise interferon γ (IFNγ)-regulated factors, *e.g.*, several enzymes of the kynurenine pathway including indoleamine 2,3-dioxygenase 1 (IDO1), immunity-related GTPases, guanylate-binding proteins and chemokines (Fig 16). In addition, we found significant perturbation of TLR, MAPK and JAK-STAT-mediated signaling cascades. Based on these results, we were able to discern many hypotheses, some of which have already been validated.

Of note is the induction of tryptophan degradation *via* the kynurenine pathway, which is usually deemed as one of the innate host immune responses to deprive and kill pathogens like *T. gondii*, because they are unable to produce tryptophan and depend on the host cell to supply this vital nutrient [113,114]. Contrary to the reported anti-parasitic function, we observed that inhibition of the rate-limiting enzyme of the kynurenine pathway (IDO1) by pharmacological and genetic means impairs the oocyst output, revealing an unforeseen supportive role of IDO1-mediated tryptophan catabolism *in vivo* (*Appendix O-P*). Indeed, we found that a byproduct of the kynurenine pathway, xanthurenic acid, can entirely reinstate the oocyst output in mice with impaired amino acid degradation. Xanthurenic acid is generally used as a gametocyte-activating factor in cultures of *Plasmodium* species [115–117]. It is presumed to foster the sexual development of *Plasmodium* by promoting exflagellation of the male gametes in mosquitoes. A similar role in gametogenesis of *E. falciformis* is also expected. Exploitation of host tryptophan catabolism by both parasites therefore appears to be conserved, despite their notably divergent primary hosts (vertebrate/invertebrate).

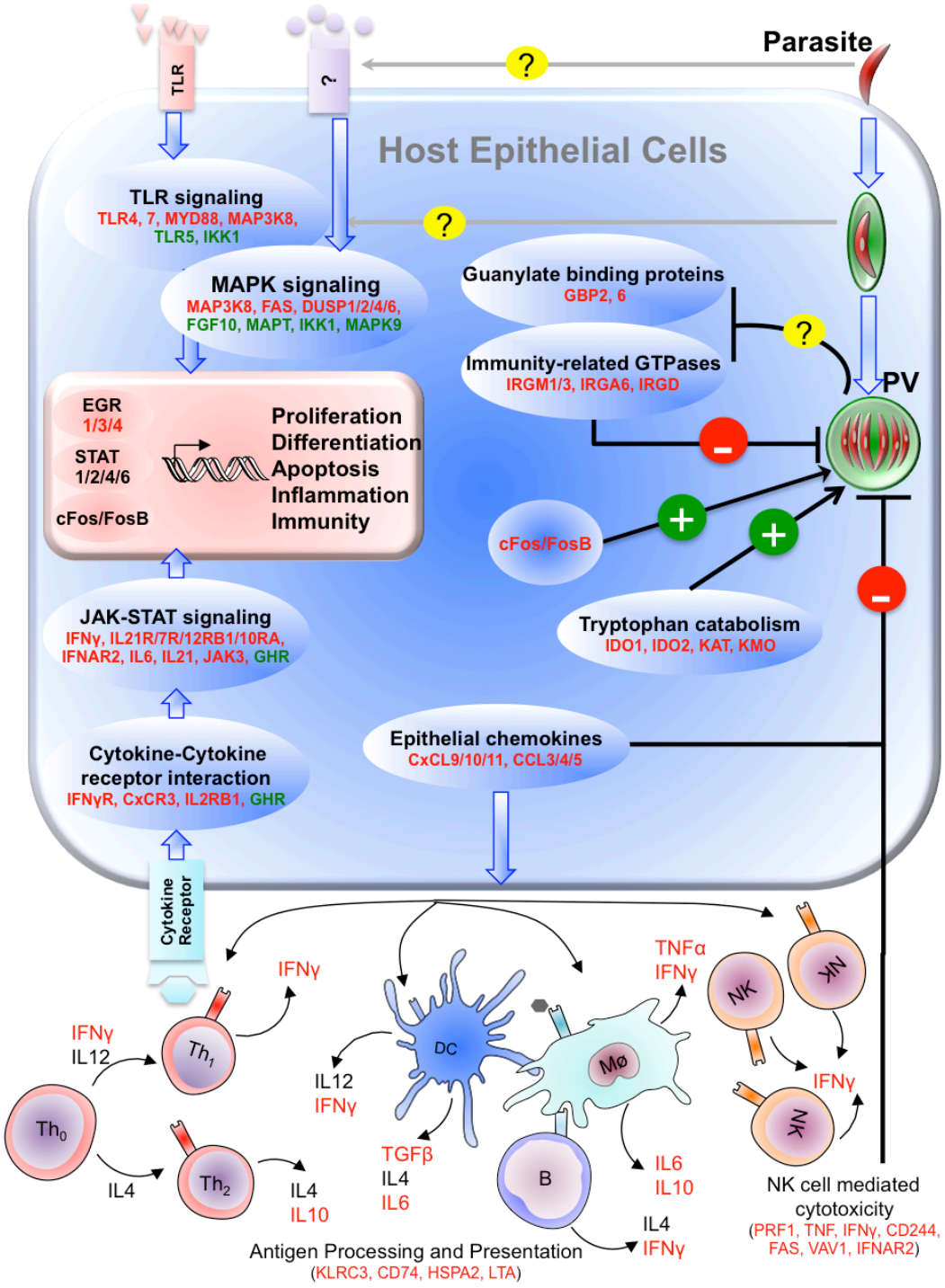

Fig 16: *Proposed model of Eimeria falciformis-mouse interactions*. The model is based on our *ex vivo* microarray analyses of the parasite-infected mouse caecum cells (except for cFoS/FosB, which are induced *in vitro*). Only those host pathways for which transgenic animals are available are depicted. The color-coded (red or green) proteins were induced or repressed. The '+' and '-' signs in circles denote a positive or negative impact of a given pathway on the parasite development, as confirmed by validation assays. Note that we observed significant modulation of immune genes, which is due to the presence of immune cells in caeca samples (enriched in epithelial cells) prepared for microarray analyses.

Our results also demonstrate other IFNγ-regulated defense mechanisms in the *Eimeria*-infected mouse including several members of the IRG and GBP family (*Appendix O*). These proteins confer resistance to a variety of organisms in a mostly pathogen-specific manner [118]. The mechanism of their action has been extensively characterized during *T. gondii* infection [119–121]. Many members of the IRG and GBP family assemble at the PVM, and afterwards kill the susceptible parasite strains. The hypervirulent strains of *T. gondii* can inactivate IRG and GBP proteins and thereby prevent their recruitment onto the PVM [122,123]. Our work found that one of the key members of the IRG family, IRGB6, failed to coat the PVM of *E. falciformis*, implying either a lack of vacuolar propensity to bind IRG, or inactivation by the parasite. Determining the precise roles of highly induced IRG and GBP proteins during *Eimeria* infection warrants further research.

In addition to the mentioned cell-intrinsic defense pathways, we observed the onset of adaptive immunity (Fig 16). The key chemokines, CxCL9-11, likely secreted by infected epithelial cells, were induced within 24 h of infection. Consistently, we saw significant increase in the chemokine-directed recruitment of lymphocytes and macrophages to the *Eimeria*-infected caeca. Inhibition of CxCR3 (a common receptor for CxCL9-11 expressed on immune cells) in parasitized animals promoted the parasite development (*Appendix O*). These data suggest a critical role of epithelial chemokines in initiating the adaptive immune response and controlling the parasite infection. Although pending validation in transgenic mice, our findings resonate well with a role of CxCR3 in orchestrating the innate and adaptive immune response against *T. gondii* tachyzoites [124].

To consolidate above *ex vivo* gene expression datasets, we executed transcriptomic analyses of the young adult mouse colon epithelial cells infected with *E. falciformis* or *T. gondii*. As expected, we detected a series of host defense pathways, such as TLR, MAPK and JAK-STAT signaling and cytokine-receptor interactions, majority of which are governed by IFNγ in response to infection regardless of the parasite in question. Surprisingly however, we observed distinct host response in many housekeeping pathways (mTOR, WNT, cAMP signaling), which implied that even related pathogens reprogram an otherwise-shared intracellular niche in a tailored manner (*Appendix Q*). Our analyses revealed only two genes (encoding cFos and Rab24 proteins) that were consistently induced by both parasites at early and late time points. Rab24 belongs to a small GTPase family possibly involved in autophagy, whereas cFos is a well-known master transcription factor (part of AP1 complex) and a proto-oncogene, which governs a repertoire of cellular processes, including but not limited to, proliferation, apoptosis, inflammation and oncogenesis. In-depth phenotyping of *T. gondii* cultures in mouse embryonic fibroblasts lacking cFos expression showed that the protein was indeed needed for an efficient intracellular replication. Plaque assays using the cFos$^{-/-}$ mutant cells confirmed an apparently essential function of cFos for the lytic cycle of *T. gondii*. Accordingly, the asexual development of *E. falciformis* was also significantly impaired in the cFos-knockout host cells. Taken together, our *ex vivo* and *in vitro* gene expression analyses support a model with divergent roles of mouse proteins during infection, some of which defend the insult mounted by the parasite, whereas others are co-opted or even subverted by the parasite.

2.5 Regulation of asexual reproduction in *Toxoplasma gondii*

Underlying publications (for abstracts, please refer to Section 5 on page 69-71):

Dynein Light Chain 8a of *Toxoplasma gondii*, a unique conoid-localized β-strand-swapped homodimer, is required for an efficient parasite growth. Qureshi B, Hoffmann N, Arroyo-Olarte RD, Nickl B, Höhne W, Jungblut P, Lucius R, Scheerer P, Gupta N; *FASEB J*, 2013, 27(3): 1034-47 **Appendix R** [125]

Optogenetic modulation of an adenylate cyclase in *Toxoplasma gondii* demonstrates a requirement of parasite cAMP for host-cell invasion and stage differentiation. Hartmann A, Arroyo-Olarte RD, Imkeller K, Hegemann P, Lucius R, Gupta N; *Journal of Biological Chemistry*, 2013, 288(19): 13705-17 **Appendix S** [126]

Toxoplasma gondii cAMP-dependent protein kinase subunit 3 is involved in the switch from tachyzoite to bradyzoite development. Sugi T, Ma YF, Tomita T, Murakoshi F, Eaton MS, Yakubu R, Han B, Tu V, Kato K, Kawazu SI, Gupta N, Suvorova ES, White MW, Kim K, Weiss LM; *mBio*, 2016, 7(3): e00755-16 **Appendix T** [127]

With a prospective aim to determine the core regulation of asexual reproduction in *T. gondii*, we have begun studying certain conserved proteins and pathways that are known to govern growth and maintenance by altering gene expression, metabolism, ion homeostasis, catalytic modulation, transport activity and protein-protein interactions. These include cNMP- and mTOR-mediated signaling and dyneins. It is noteworthy that *Toxoplasma* harbors one of the largest repertoires of dynein light chains (DLC). DLC8 is one such common eukaryotic protein, which controls diverse functions, most notably cargo transport and protein-protein interactions. We show that parasites harbor 4 DLC8 proteins (*Tg*DLC8a-d), of which only *Tg*DLC8a clusters in the archetypal DLC8 class. *Tg*DLC8b-d proteins form a divergent alveolate-specific clade (*Appendix R*). *Tg*DLC8b-d proteins are mainly cytosolic, whereas *Tg*DLC8a resides in the conoid region of *T. gondii*. Quite intriguingly, the apical location of *Tg*DLC8a is not shared by its nearly identical orthologs from *Plasmodium* and *Eimeria* species. Unlike *Pf*DLC8, but akin to *Hs*DLC8, *Tg*DLC8a exhibits a *classic* homodimeric structure with two identical binding grooves, which are designed for multi-target recognition (Fig 17). These results show a surprising structural-functional divergence of the two otherwise-conserved proteins from related parasites.

One of the well-known functions of dyneins is the minus-end-directed retrograde cargo transport in mammalian cells [128]. Notably, the minus ends of the spiral and subpellicular microtubules in *T. gondii* are located in the conoid region, which implies a function of *Tg*DLC8a in the minus-end-directed transport of the protein cargo to the parasite apex, probably along the pair of intra-conoid microtubules. This notion resonates with the apical presence of *Tg*DLC8a in tachyzoites. Yet another elemental role of mammalian DLC8 is to promote dimerization/stabilization of the interacting partners and thereby regulate several subcellular events [129]. Indeed, we found that the dimerization of *Tg*DLC8a is a prerequisite for efficient growth of tachyzoites. Further studies identified elongation factor 1α/2, glycine-rich protein 2, glutamate-rich protein, dyneins and few

hypothetical proteins as putative interacting partners of *Tg*DLC8a, which point to a central role of homodimer in regulating motor as well as non-motor processes. Our focus now is to define the specific functions and physiological impact of *Tg*DLC8a and other related proteins in *T. gondii*.

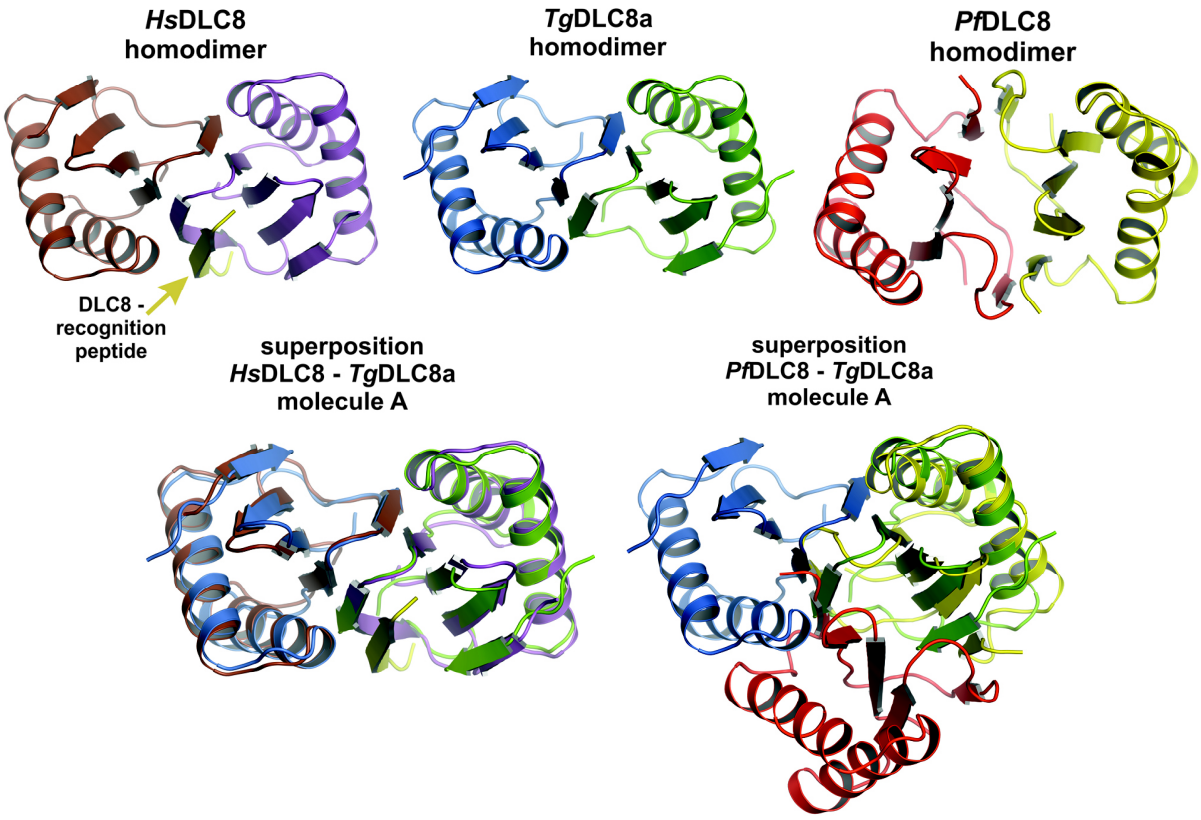

Fig 17: *Comparison of TgDLC8a with HsDLC8 and PfDLC8 structures. Top row*: The monomer-monomer crystal packing of *Hs*DLC8 (brown and purple), *Tg*DLC8a (blue and green) and *Pf*DLC8 (red and yellow) homodimers. The yellow arrow indicates the co-crystallized recognition peptide of *Hs*DLC8. *Bottom row*: Superimposition of the monomer subunits from *Tg*DLC8a and *Hs*DLC8 counterparts based on C_α atoms yielded a root mean square deviation of 1.0Å. In contrast, superposition of the monomer subunits from *Tg*DLC8a and *Pf*DLC8 counterparts yielded a much higher root mean square deviation (2.4Å).

Similar to dynein light chains, cyclic nucleotides-mediated signaling is expected to govern several parasite processes, *e.g.*, lytic cycle and stage differentiation. However, there has been no evidence establishing a direct relationship between the activation of the parasite-specific signaling cascade and downstream effects because the experimental modulation of cAMP and cGMP in parasites has so far relied on chemical modulators that were initially developed to target mammalian cells. A chemical approach is suitable for extracellular stages, however not quite for intracellular stages enclosed within a mammalian cell, which is perturbed at the same time. To circumvent the issue, we conceived optogenetic induction of cyclic nucleotide signaling in *T. gondii* (*Appendix S*). Ectopic expression of a bacterial photo-activated adenylate cyclase in *T. gondii* allowed a light-sensitive control of cAMP within the parasite cytosol. This study also revealed a role of the parasite cAMP

in tachyzoite-bradyzoite conversion and stage-specific gene expression (Fig 18). We showed that a transient induction of cytosolic cAMP within the parasite promotes cyst formation, whereas a prolonged pulse inhibits this process. We are currently establishing an optimized optogenetically-regulated system, which would allow us to examine the metabolic, epigenetic and transcriptional rewiring in response to cAMP-mediated bidirectional stage switching in *T. gondii*. Such a model shall be valuable to investigate the mechanisms of latency and recrudescence during the parasite infection, the latter of which remains unequivocally enigmatic till date.

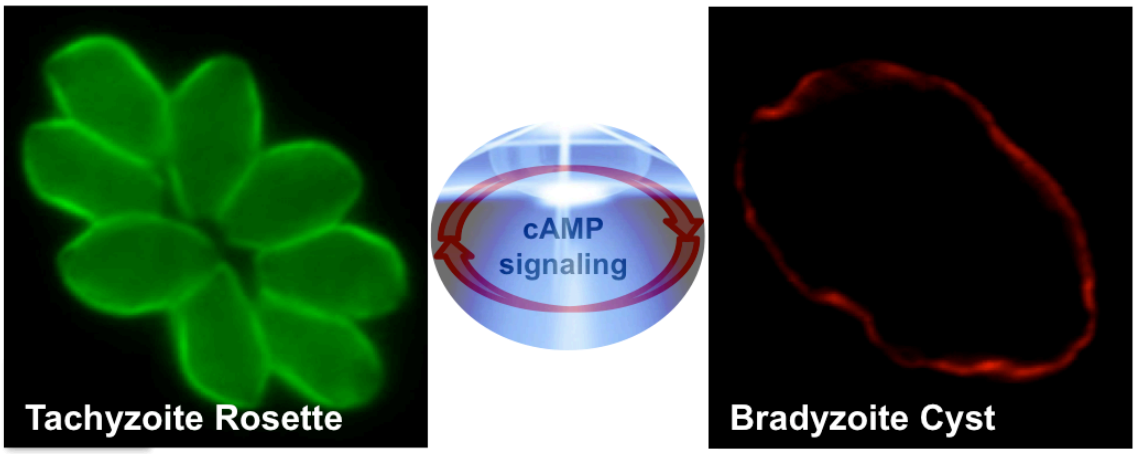

Fig 18: *Optogenetic induction cAMP-mediated stage differentiation in T. gondii*. A light-activated adenylate cyclase originally isolated from a lithotropic bacterium *Beggiatoa* was expressed in a cyst-forming strain of *T. gondii*. Cultures were exposed to blue light, which induces cAMP-dependent stage differentiation in tachyzoites. Tachyzoite (green) and bradyzoite (red) stages were visualized by immunostaining of the stage-specific *Tg*Sag1 and *Tg*CST1 proteins, respectively.

Our parallel work has characterized the native cAMP and cGMP pathways in *T. gondii*. We have identified most of the central mediators present in the parasite, which include at least 4 adenylate cyclases (AC1-4), 3 cAMP-dependent kinases (catalytic subunits PKAc1-3 & regulatory subunits PKAr1-3), 1 guanylate cyclase, 1 cGMP-dependent kinase (PKG) and 15 phosphodiesterases. Initial data indicate an essential nature of cGMP signaling and partly redundant cAMP signaling in the hypervirulent strain of *T. gondii*. In a collaborative study, we examined how cAMP controls the process of parasite differentiation (*Appendix T*). This work revealed that unlike *Tg*PKAc1 and *Tg*PKAc2, which are conserved in the phylum apicomplexa, *Tg*PKAc3 appears evolutionarily divergent and specific to coccidians. *Tg*PKAc3 was genetically ablated in the cyst-forming as well as hypervirulent strains of *T. gondii*. The Δ*pkac3* mutant exhibited slower growth that correlated with a higher basal rate of bradyzoite formation, which suggests a role of *Tg*PKAc3 in cAMP-dependent maintenance of the cell cycle. The Δ*pkac3* mutant also had a defect in producing brain cysts, indicating that a substrate of *Tg*PKAc3 probably regulates the persistence in the rodent host. We are currently exploring other mediators of cNMP cascades by gene deletion and phenotyping of the mutants.

3 CONCLUSIONS AND PERSPECTIVES

This study aimed to appreciate the functioning and impact of metabolic networks in intertwined host-parasite models. We strived to fathom the relationships between the metabolic capacities of selected parasites, their ability to exploit host metabolism and survive nutritional perturbations, which provide clues as to how distinct parasites have evolved with intracellular niches and how to inhibit them selectively. Indeed, our work on *T. gondii*, *P. berghei* and *E. falciformis* has proven fairly complementary to resolve a number of inter-related premises. In addition, a comparison of their metabolic designs with mammalian cells has identified vulnerabilities within parasites' networks. At least, three paradigms emerge from this work, which have potential to advance our knowledge even further. 1) Metabolic plasticity and determinants of intracellular parasitism; 2) Physiological convergence between parasites and tumor cells; 3) Utility of optogenetics in infection research

3.1 Metabolic plasticity and determinants of intracellular parasitism

A genome-wide comparison of enzymes and transporters suggested reduced metabolic potential and generally host-dependent lifestyles of indicated parasites. Nonetheless, they harbor a nearly complete carbon-metabolism framework to produce macromolecules using host-derived carbon sources. We determined that glucose and glutamine together furnish a major fraction of biomass and energy in a highly co-regulated manner, and such a cooperative metabolism is vital for the lytic cycle of *T. gondii* tachyzoites in mammalian cells. Tachyzoites are also competent in utilizing acetate when available, which can rescue the modest growth defect in glycolysis-deficient mutant. Unexpectedly, tachyzoites continue to replicate without any of the three primary carbon sources, which reflects an exceptional metabolic plasticity, given the facts they are subjected to reductive evolution and adapted to strictly intracellular parasitism. Our ongoing work suggests a number of other host nutrients exploited by intracellular tachyzoites, especially when the major carbon sources become limited. A versatile carbon metabolism with unprecedented nutritional flexibility is anticipated to ensure the survival of *T. gondii* in diverse host-cell types.

Apicomplexans are also competent in producing major phospholipids for membrane biogenesis, which is compartmentalized, interlinked, mutually regulated and partially redundant. We reveal that *Toxoplasma* tachyzoites as well as *Eimeria* sporozoites can utilize host-derived and endogenous precursors to manufacture all standard phospholipids, namely PtdCho, PtdEtn, PtdIns, PtdSer, PtdGro and PtdOH. Besides, they express some exclusive parasite-specific lipids (PtdThr, EPC, IPC), which likely contribute to their specific lifestyle features not shared with mammalian cells. Noticeably, lipid biogenesis in both parasites is a patchwork of prokaryotic- and eukaryotic-type pathways. Similar to the central carbon metabolism, tachyzoites exhibit a remarkable plasticity in phospholipid biogenesis, which include compositional flexibility of membranes, partly redundant routes of PtdEtn synthesis and rapid resilience of mutants to genetic or biochemical interference. Such characteristics may enable *T. gondii* tachyzoites to fine-tune membrane synthesis according to intracellular milieus encountered in a wide rage of host organisms and plausibly contribute to its evolution as a promiscuous pathogen.

Of note is also the comparison of sugar metabolism in *Toxoplasma* and *Plasmodium*, which exhibit a striking divergence in carbon metabolism. *Plasmodium* species lack gluconeogenesis, which renders glycolysis essential. The pan-hexose permease appears to be indispensable for all developmental stages of *Plasmodium* and thus offers an attractive drug target. Our *Pf*HT1-transgenic yeast and *P. berghei* models for screening and pharmacological assessment of analog-based drugs shall be useful for preclinical evaluation of antimalarials. Physiological essentiality of phylogenetically divergent metabolic pathways and certain auxotrophies in parasites provide excellent therapeutic avenues. Our *proof-of-concept* study already demonstrated selective disruption of the lytic cycle by inhibiting *de novo* synthesis of PtdCho as well as the *vaccination* potential of metabolically attenuated PTS and CDS mutants to prevent toxoplasmosis. Collectively, these data underline the translational value of our research. Last but not least, our involvement with *E. falciformis* has identified and validated several host factors determining the parasite development. We show how IFNγ signaling plays opposing roles during infection and how *Eimeria* subverts a conserved metabolic pathway of its rodent host. This work holds promise to study the impact of host pathways on the entire lifecycle of a prevalent coccidian parasite. It is now timely to reconstruct mathematical models of parasite-host metabolic interactions to deduce the network design principles of parasitism.

3.2 Physiological convergence between parasites and cancer cells

Over the last decade, a consistent picture of carbon metabolism has emerged from studies on diverse types of proliferating cells, whose metabolic requirements are different from differentiated (quiescent) cells. For instance, cancer cells must continually produce the major constituents of biomass (nucleic acid, protein, membrane). Most differentiated cells however are relieved of such liabilities and reduce their carbon flux to promote maintenance and survival. Metabolic demands of differentiated cells are primarily met by glucose, most of which (>90%) enters the TCA cycle as pyruvate and only a small fraction (<10%) is converted to lactate. Most dividing cells instead require rapid glycolytic flux concurrent with the excretion of lactate, and substantial catabolism of glutamine and acetate. At first glance, such a carbon flux seems rather inefficient for making ATP and the waste of three carbons as lactate; nonetheless, it confers much-needed benefits to dividing cells by rerouting glycolytic and TCA cycle metabolites for making biomass.

Intracellular parasites undergoing exponential growth exhibit a cancer-like metabolism, whereas extracellular parasites (non-dividing state) appear to depend on the oxidative metabolism, which corresponds to differentiated mammalian cells. It therefore appears as though parasites and host cells have similar metabolic phenotypes when faced with alike biological fate. This conservation implies that there is an evolutionary advantage to non-oxidative metabolism during proliferation and to oxidative metabolism during quiescence. Our notion that physiological cooperativity and metabolic quintessence of the major carbon sources in *T. gondii* tachyzoites resembles tumor cells may eventually be exploited to develop common therapeutics against both vices. The unicellular parasites might also provide pertinent working models to recognize the evolution of proliferative metabolism in eukaryotes. A systematic co-modeling of the parasite and cancer cells for example can discern the minimal metabolic framework and evolutionary origin of Warburg Effect. Since

the lytic cycle comprises periodic switches between the intracellular proliferative and extracellular non-proliferative stages, working with a parasite model (*e.g.*, tachyzoite) may also reveal metabolic reprogramming and minimum-essential regulation of proliferation *versus* quiescence in eukaryotic cells. Our work therefore potentially bridges the disciplines of parasitology and tumor biology – the consequences of which have yet to be seen.

3.3 Utility of optogenetics in infection research

The recent success of optogenetics in neurobiology prompted us to establish its initial application in infection research using *T. gondii* as a model pathogen. Specially, the study of cyclic nucleotides signaling in *T. gondii* has proven difficult due to absence of parasite-specific methods and required a *nonconventional* optogenetic approach. Indeed, we were successful in defining many physiological roles of the parasite-derived cAMP using an optogenetic actuator. We could achieve an efficient, spatial, dynamic and specific induction of the parasite signaling without influencing host cells, which has not been feasible by chemical modulators, particularly when working with intracellular parasites. Optogenetics has also bestowed other much-desired advantages, such as (*a*) reversible and quantitative modulation of second messengers, (*b*) gene-encoded activity, which is inheritable to the progeny, (*c*) circumvention of routine issues faced in parasite cultures when using chemicals (poor diffusion, premature degradation, sustained effect *etc.*).

Our founding study has already steered further work on the repression and detection of cNMP-directed signaling using photo-activated phosphodiesterases and biosensors, respectively. We also aim to resolve the ion and phosphoinositide-mediated transduction pathways using light-sensitive ion channels and enzymes. Likewise, it is quite conceivable to combine the optogenetic actuators of signaling (cNMP, calcium, phosphoinositides *etc*.) with corresponding gene-encoded sensors to photo-oscillate and monitor the processes in real-time. A similar approach using multi-parameter imaging can potentially unravel the crosstalk between the signaling cascades. The ever-increasing discoveries and tailored engineering of light-responsive proteins and color-tuned isoforms shall further widen the dimension of infection research that can be assumed. In brief, integrated utility of optogenetics, cell biology and reverse genetics are expected to turn out extremely valuable in determining the biological importance of secondary messengers and to examine the endogenous signaling machinery in intracellular parasites. We anticipate such a progressive approach will not only consolidate optogenetics in parasitology research, but also facilitate its wider application to other entwined models, such as host-pathogen and symbiotic relationships.

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5 PUBLICATION ABSTRACTS AND AUTHOR CONTRIBUTIONS

Appendix A

Martin Blume, Dayana Rodriguez-Contreras, Scott Landfear, Tobias Fleige, Dominique Soldati-Favre, Richard Lucius, Nishith Gupta
Host-derived glucose and its transporter in the obligate intracellular pathogen *Toxoplasma gondii* are dispensable by glutaminolysis. *Proceedings of National Academy of Sciences USA*, 2009, 106 (31): 12998-3003

Toxoplasma gondii, as an obligate intracellular and promiscuous pathogen of mammalian cells, utilizes host sugars for energy and to generate glycoconjugates that are important to its survival and virulence. Here, we report that *T. gondii* glucose transporter (*Tg*GT1) is proficient in transporting mannose, galactose, and fructose besides glucose, and serves as a major hexose transporter at its plasma membrane. *Toxoplasma* harbors 3 additional putative sugar transporters (*Tg*ST1-3), of which *Tg*ST2 is expressed at its surface, whereas *Tg*ST1 and *Tg*ST3 are intracellular. Surprisingly, *Tg*GT1 and *Tg*ST2 are nonessential to the parasite as their ablations inflict only a 30% or no defect in its intracellular growth, respectively. Indeed, *Toxoplasma* can also tolerate the deletion of both genes while incurring no further growth phenotype. Unlike Δ*tgst2*, the modest impairment in Δ*tggt1* and Δ*tggt1*/Δ*tgst2* mutants is because of a minor delay in their intracellular replication, which is a direct consequence of the abolished import of glucose. The Δ*tggt1* displays an attenuated motility in defined minimal media that is rescued by glutamine. *Tg*GT1-complemented parasites show an entirely restored growth, motility, and sugar import. The lack of exogenous glucose in Δ*tggt1* culture fails to accentuate its intrinsic growth defect and prompts it to procure glutamine to sustain its metabolism. Unexpectedly, *in vivo* virulence of Δ*tggt1* in mice remains unaffected. Taken together, our data demonstrate that glucose is nonessential for *T. gondii* tachyzoites, underscore glutamine is a complement substrate, and provide a basis for understanding the adaptation of *T. gondii* to diverse host cells.

I initiated this study during my postdoctoral research under the scholastic guidance of RL at Humboldt University of Berlin. It serves as the first example of my independent research, which was conceived, directed and partly executed by me. The work also constitutes parts of PhD and MS theses submitted by MB under my supervision.

Author contributions: NG and MB designed research; MB, NG, DRC and TF performed research; SL, DSF, RL and NG contributed new reagents/analytic tools; MB, NG, DSF and SL analyzed data; NG and MB wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix B

Martin Blume, Marion Hliscs, Dayana Rodriguez-Contreras, Marco Sanchez, Scott Landfear, Richard Lucius, Kai Matuschewski, Nishith Gupta
A constitutive pan-hexose permease in *Plasmodium* and models for high-throughput screening of anti-malarial sugar analogs. *FASEB J*, 2011, 25(4): 1218-29

Glucose is considered essential for erythrocytic stages of the malaria parasite, *Plasmodium falciparum*. Importance of sugar and its permease for hepatic and sexual stages of *Plasmodium*, however, remains elusive. Moreover, increasing global resistance to current antimalarials necessitates the search for novel drugs. Here, we reveal that hexose transporter 1 (HT1) of *Plasmodium berghei* can transport glucose (K_m ~87 μM), mannose (K_i ~93 μM), fructose (K_i ~0.54 mM), and galactose (K_i ~5 mM) in *Leishmania mexicana* mutant and *Xenopus laevis*; and, therefore, is functionally equivalent to HT1 of *P. falciparum* (Glc, K_m ~175 μM; Man, K_i ~276 μM; Fru, K_i ~1.25 mM; Gal, K_i ~5.86 mM). Notably, a glucose analog, C3361, attenuated hepatic (IC_{50} ~15 μM) and ookinete development of *P. berghei*. The *Pb*HT1 could be ablated during intraerythrocytic stages only by concurrent complementation with *Pb*HT1-HA or *Pf*HT1. Together; these results signify that *Pb*HT1 and glucose are required for the entire life cycle of *P. berghei*. Accordingly, *Pb*HT1 is expressed in the plasma membrane during all parasite stages. To permit a high-throughput screening of *Pf*HT1 inhibitors and their subsequent *in vivo* assessment, we have generated *Saccharomyces cerevisiae* mutant expressing codon-optimized *Pf*HT1, and a *Pf*HT1-dependent Δ*pbht1* parasite strain. This work provides a platform to facilitate the development of drugs against malaria, and it suggests a disease-control aspect by reducing parasite transmission.

I conceived the study, which was then designed and implemented with the support of coauthors. MB, MH, DRC and MS generated most datasets. The work was performed within the framework of PhD dissertation submitted by MB under my supervision.

Author contributions: NG and MB designed research; MB, MH, DRC, and MS performed research; KM, SL, RL and NG contributed new reagents/analytic tools; MB, NG and KM analyzed data; NG and MB wrote the paper. All authors approved the manuscript.

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Appendix C

Richard Nitzsche, Vyacheslav Zagoriy, Richard Lucius, Nishith Gupta
Metabolic cooperation of glucose and glutamine is essential for the lytic cycle of obligate intracellular parasite *Toxoplasma gondii*. *Journal of Biological Chemistry*, 2016, 291(1): 126-41

Toxoplasma gondii is a widespread protozoan parasite infecting nearly all warm-blooded organisms. Asexual reproduction of the parasite within its host cells is achieved by consecutive lytic cycles, which necessitates biogenesis of significant energy and biomass. Here we show that glucose and glutamine are the two major physiologically important nutrients used for the synthesis of macromolecules (ATP, nucleic acid, proteins, and lipids) in *T. gondii*, and either of them is sufficient to ensure the parasite survival. The parasite can counteract genetic ablation of its glucose transporter by increasing the flux of glutamine-derived carbon through the tricarboxylic acid cycle and by concurrently activating gluconeogenesis, which guarantee a continued biogenesis of ATP and biomass for host-cell invasion and parasite replication, respectively. In accord, a pharmacological inhibition of glutaminolysis or oxidative phosphorylation arrests the lytic cycle of the glycolysis-deficient mutant, which is primarily a consequence of impaired invasion due to depletion of ATP. Unexpectedly, however, intracellular parasites continue to proliferate, albeit slower, notwithstanding a simultaneous deprivation of glucose and glutamine. A growth defect in the glycolysis-impaired mutant is caused by a compromised synthesis of lipids, which cannot be counterbalanced by glutamine but can be restored by acetate. Consistently, supplementation of parasite cultures with exogenous acetate can amend the lytic cycle of the glucose transport mutant. Such plasticity in the parasite's carbon flux enables a growth-and-survival trade-off in assorted nutrient milieus, which may underlie the promiscuous survival of *T. gondii* tachyzoites in diverse host cells. Our results also indicate a convergence of parasite metabolism with cancer cells.

This work was conceived by myself, and designed together with RN and VZ. RN did most of the experimentation and compiled results into a manuscript with my support. The work also constitutes a part of PhD dissertation (to be submitted by RN) under my mentorship.

Author contributions: NG conceived, designed, and coordinated the study, analyzed experiments, and wrote the paper; RN and VZ designed, performed, and analyzed the experiments and wrote the paper; RL contributed analytical tools/reagents. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix D

Martin Blume, Richard Nitzsche, Ulrich Sternberg, Motti Gerlic, Seth L Masters, Nishith Gupta, Malcolm J McConville
A *Toxoplasma gondii* gluconeogenic enzyme contributes to robust central carbon metabolism and is essential for replication and virulence. *Cell Host & Microbe*, 2015, 18(2): 210-20

The expression of gluconeogenic enzymes is typically repressed when glucose is available. The protozoan parasite *Toxoplasma gondii* utilizes host glucose to sustain high rates of intracellular replication. However, despite their preferential utilization of glucose, intracellular parasites constitutively express two isoforms of the gluconeogenic enzyme fructose 1,6-bisphosphatase (*Tg*FBP1 and *Tg*FBP2). The rationale for constitutive expression of FBPases in *T. gondii* remains unclear. We find that conditional knockdown of *Tg*FBP2 results in complete loss of intracellular growth *in vitro* under glucose-replete conditions and loss of acute virulence in mice. *Tg*FBP2 deficiency was rescued by expression of catalytically active FBPase and was associated with altered glycolytic and mitochondrial TCA cycle fluxes, as well as dysregulation of glycolipid, amylopectin, and fatty acid biosynthesis. Futile cycling between gluconeogenic and glycolytic enzymes may constitute a regulatory mechanism that allows *T. gondii* to rapidly adapt to changes in nutrient availability in different host cells.

This study originates from the PhD work of MB under my supervision. It was conceptualized and initiated by MB and myself at HUB, and continued by MB in MJM's laboratory at the University of Melbourne. RN and US performed additional work needed to ensure the publication while working under my mentorship.

Author contributions: MB, MJM, NG, MG and SLM designed the study; MB, RN, US and MG performed assays; MB, MJM and NG wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix E

Richard Nitzsche, Maximilian Tischer, Vyacheslav Zagoriy, Nishith Gupta
A mitochondrial phosphoenolpyruvate carboxykinase ensures glucose-independent survival of the protozoan parasite *Toxoplasma gondii* (*submitted*)

Toxoplasma gondii is considered as one of the most successful intracellular pathogens, because it can reproduce in varied nutritional milieus, encountered in diverse host-cell types of essentially any warm-blood organism. Our earlier work has demonstrated that the acute (tachyzoite) stage of *T. gondii* depends on cooperativity of glucose and glutamine catabolism to sustain its carbon demands and underlying metabolic plasticity. The mechanism of glutamine flux and its genetic importance in the absence of glucose catabolism remained to be established. Here, we reveal two phylogenetically distinctive phosphoenolpyruvate carboxykinase (PEPCK) in the parasite, one of which localizes in the mitochondrion ($PEPCK_{mt}$), whereas the other protein is not expressed in tachyzoites ($PEPCK_{net}$). Parasites can tolerate genetic deletion of $PEPCK_{mt}$ as well as $PEPCK_{net}$, indicating nonessential roles of the two isoforms in standard glucose-replete cultures. Moreover, $PEPCK_{net}$, but not $PEPCK_{mt}$, could also be ablated in glycolysis-deficient mutant, which implied a critical function of the latter enzyme in glucose-independent survival of tachyzoites. In accord, the lytic cycle of a conditional mutant of $PEPCK_{mt}$ in the glycolysis-impaired strain was aborted following tetracycline-induced repression of the enzyme. Isotope-resolved metabolomics analysis of the conditional mutant displayed rather selective defects in glutamine flux into gluconeogenesis and pentose phosphate pathway, both of which are required to ensure biomass production in the absence of glucose utilization. In addition, our data suggest an anaplerotic role of $PEPCK_{mt}$ that enables parasites to interlink glycolysis and TCA cycle under normal glucose-replete conditions. Conversely, we found that otherwise-anaplerotic enzyme pyruvate carboxylase is dispensable not only in glycolysis-competent strain but also in glycolysis-deficient mutant even though it localizes in the parasite mitochondrion. Last but not least, the observed physiology of tachyzoites appears to phenocopy cancer cells when faced with similar nutritional environments. Such a metabolic convergence holds promise for the development of common therapeutics against both threats.

This study constitutes an extension of RN's previous work (Appendix C). I conceptualized the research and designed it together with RN. The work also constitutes the MS thesis of MT and PhD dissertation of RN (to be submitted) under my mentorship.

Author contributions: NG conceived the study; NG and RN designed the work; RN, MT and VZ performed research; VZ and NG contributed reagents/analytic tools; RN, VZ and NG analyzed data; RN and NG wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the submission of this work.

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Appendix F

Nishith Gupta, Matthew M Zahn, Isabelle Coppens, Keith A Joiner, Dennis R Voelker
Selective disruption of phosphatidylcholine metabolism of the intracellular parasite *Toxoplasma gondii* arrests its growth. *Journal of Biological Chemistry*, 2005, 280(16): 16345–53

Toxoplasma gondii is an intracellular protozoan parasite capable of causing devastating infections in immunocompromised and immunologically immature individuals. In this report, we demonstrate the relative independence of *T. gondii* from its host cell for aminoglycerophospholipid synthesis. The parasite can acquire the lipid precursors serine, ethanolamine, and choline from its environment and use them for the synthesis of its major lipids, phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho), respectively. Dimethylethanolamine ($Etn(Me)_2$), a choline analog, dramatically interfered with the PtdCho metabolism of *T. gondii* and caused a marked inhibition of its growth within human foreskin fibroblasts. In tissue culture medium supplemented with 2 mm $Etn(Me)_2$, the parasite-induced lysis of the host cells was dramatically attenuated, and the production of parasites was inhibited by more than 99%. The disruption of parasite growth was paralleled by structural abnormalities in its membranes. In contrast, no negative effect on host cell growth and morphology was observed. The data also reveal that the $Etn(Me)_2$-supplemented parasite had a time-dependent decrease in its PtdCho content and an equivalent increase in phosphatidyldimethylethanolamine, whereas other major lipids, PtdSer, PtdEtn, and PtdIns, remained largely unchanged. Relative to host cells, the parasites incorporated more than 7 times as much $Etn(Me)_2$ into their phospholipid. These findings reveal that $Etn(Me)_2$ selectively alters parasite lipid metabolism and demonstrate how selective inhibition of PtdCho synthesis is a powerful approach to arresting parasite growth.

The study stems from my own work performed during the first postdoctoral position under the scholastic supervision of DRV at National Jewish Medical Research Center (Denver). The work was conceived and designed by DRV and myself. I executed most of the assays and interpreted the data, occasionally aided by MMZ and IC.

Author contributions: NG and DRV designed research; NG, MMZ and IC performed research; IC, KAJ and DRV contributed new reagents/analytic tools; NG and DRV analyzed data; NG and DRV wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix G

Vera Sampels, Anne Hartmann*, Isabelle Dietrich, Isabelle Coppens, Lilach Sheiner, Boris Striepen, Andreas Herrmann§, Richard Lucius, Nishith Gupta
Conditional mutagenesis of a novel choline kinase demonstrates the plasticity of phosphatidylcholine biogenesis and gene expression in *Toxoplasma gondii*. *Journal of Biological Chemistry*, 2012, 287(20): 16289-99

The obligate intracellular and promiscuous protozoan parasite *Toxoplasma gondii* needs an extensive membrane biogenesis that must be satisfied irrespective of its host-cell milieu. We show that the synthesis of the major lipid in *T. gondii*, phosphatidylcholine (PtdCho), is initiated by a novel choline kinase (*Tg*CK). Full-length (≈70-kDa) *Tg*CK displayed a low affinity for choline (K_m ≈0.77 mM) and harbors a unique N-terminal hydrophobic peptide that is required for the formation of enzyme oligomers in the parasite cytosol but not for activity. Conditional mutagenesis of the *Tg*CK gene in *T. gondii* attenuated the protein level by ≈60%, which was abolished in the *off state* of the mutant ($\Delta tgck_i$). Unexpectedly, the mutant was not impaired in its growth and exhibited a normal PtdCho biogenesis. The parasite compensated for the loss of full-length *Tg*CK by two potential 53- and 44-kDa isoforms expressed through a cryptic promoter identified within exon 1. *Tg*CK-Exon1 alone was sufficient in driving the expression of GFP in *E. coli*. The presence of a cryptic promoter correlated with the persistent enzyme activity, PtdCho synthesis, and susceptibility of *T. gondii* to a choline analog, dimethylethanolamine. Quite notably, the mutant displayed a regular growth in the *off state* despite a 35% decline in PtdCho content and lipid synthesis, suggesting a compositional flexibility in the membranes of the parasite. The observed plasticity of gene expression and membrane biogenesis can ensure a faithful replication and adaptation of *T. gondii* in disparate host or nutrient environments.

This work is an extension of my postdoctoral research performed in Denver (Appendix F), which was continued when I began my independent work at HUB. My former PhD (VS, AH) and master (ID) students performed most of the experimental work.*

Author contributions: NG conceived the study; NG and VS designed research; VS, AH*, ID and NG performed research; IC, LS, BS, AH§ and RL contributed reagents and/or analytic tools; NG, VS and IC analyzed data; NG and VS wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix H

Nishith Gupta, Anne Hartmann, Richard Lucius, Dennis R Voelker
The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase. *Journal of Biological Chemistry*, 2012, 287(27): 22938-47

Toxoplasma gondii is an obligate intracellular parasite capable of causing fatal infections in immunocompromised individuals and neonates. Examination of the phosphatidylserine (PtdSer) metabolism of *T. gondii* reveals that the parasite secretes a soluble form of PtdSer decarboxylase (*Tg*PSD1), which preferentially decarboxylates liposomal PtdSer with an apparent K_m of 67 μM. The specific enzyme activity increases by 3-fold during the replication of *T. gondii*, and soluble phosphatidylserine decarboxylase (PSD) accounts for ≈20% of the total PSD, prior to the parasite egress from the host cells. Extracellular *T. gondii* secreted ≈20% of its total PSD activity at 37°C, and the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) inhibited the process by 50%. Cycloheximide, brefeldin A, ionic composition of the medium, and exogenous PtdSer did not modulate the enzyme secretion, which suggests a constitutive discharge of a presynthesized pool of PSD in axenic *T. gondii*. *Tg*PSD1 consists of 968 amino acids with a 26-amino acid hydrophobic peptide at the N terminus and no predicted membrane domains. Parasites overexpressing *Tg*PSD1-HA secreted 10-fold more activity compared with the parental strain. Exposure of apoptotic Jurkat cells to transgenic parasites demonstrated interfacial catalysis by secreted *Tg*PSD1 that reduced host cell surface exposure of PtdSer. Immunolocalization experiments revealed that *Tg*PSD1 resides in the dense granules of *T. gondii* and is also found in the parasitophorous vacuole of replicating parasites. Together, these findings demonstrate novel features of the parasite enzyme because a secreted, soluble, and interfacially active form of PSD has not been previously described for any organism.

This study originates from the experimental work performed during my first postdoctoral position under the guidance of DRV at National Jewish Medical Research Center (Denver). The work was conceived and designed by myself with DRV's support. I generated most datasets and drafted a manuscript for submission while working in Denver, which required additional assays done by AH to ensure the publication. AH has reported her contribution in a PhD dissertation under my mentorship.

Author contributions: NG and DRV designed research; NG and AH performed research; DRV and RL contributed new reagents and analytical tools; NG and AH analyzed data; NG wrote the paper. All authors approved the manuscript.

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Appendix I

Anne Hartmann, Maria Hellmund, Richard Lucius, Dennis R Voelker, Nishith Gupta
Phosphatidylethanolamine synthesis in the parasite mitochondrion is required for efficient growth but dispensable for survival of *Toxoplasma gondii*. *Journal of Biological Chemistry*, 2014, 289(10): 6809-24

Toxoplasma gondii is a highly prevalent obligate intracellular parasite of the phylum Apicomplexa, which also includes other parasites of clinical and/or veterinary importance, such as *Plasmodium*, *Cryptosporidium*, and *Eimeria*. Acute infection by *Toxoplasma* is hallmarked by rapid proliferation in its host cells and requires significant synthesis of parasite membranes. Phosphatidylethanolamine (PtdEtn) is the second major phospholipid class in *T. gondii*. Here, we reveal that PtdEtn is produced in the parasite mitochondrion and parasitophorous vacuole by decarboxylation of phosphatidylserine (PtdSer) and in the endoplasmic reticulum by fusion of CDP-ethanolamine and diacylglycerol. PtdEtn in the mitochondrion is synthesized by a phosphatidylserine decarboxylase (*Tg*PSD1$_{mt}$) of the type I class. *Tg*PSD1$_{mt}$ harbors a targeting peptide at its N terminus that is required for the mitochondrial localization but not for the catalytic activity. Ablation of *Tg*PSD1$_{mt}$ expression caused up to 45% growth impairment in the parasite mutant. The PtdEtn content of the mutant was unaffected, however, suggesting the presence of compensatory mechanisms. Indeed, metabolic labeling revealed an increased usage of ethanolamine for PtdEtn synthesis by the mutant. Likewise, depletion of nutrients exacerbated the growth defect (≈56%), which was partially restored by ethanolamine. Besides, the survival and residual growth of the *Tg*PSD1$_{mt}$ mutant in the nutrient-depleted medium also indicated additional routes of PtdEtn biogenesis, such as acquisition of host-derived lipid. Collectively, the work demonstrates a metabolic cooperativity between the parasite organelles, which ensures a sustained lipid synthesis, survival and growth of *T. gondii* in varying nutritional milieus.

I conceived and supervised this work, which was executed within the framework of PhD dissertation of AH and master's thesis of MH. I also did a minor part of this work when working as a postdoctoral fellow in Denver.

Author contributions: NG conceived the study; NG and AH designed research; AH, MH and NG performed research; RL and DRV contributed new analytical reagents; AH and NG analyzed data and wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix J

Ruben D Arroyo-Olarte, Jos F Brouwers, Arunakar Kuchipudi, Bernd J Helms, Aindrilla Biswas, Ildiko R Dunay, Richard Lucius, Nishith Gupta
Phosphatidylthreonine and lipid-mediated control parasite virulence. *PLoS Biology*, 2015, 13(11): e1002288

The major membrane phospholipid classes, described thus far, include phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns). Here, we demonstrate the natural occurrence and genetic origin of an exclusive and rather abundant lipid, phosphatidylthreonine (PtdThr), in a common eukaryotic model parasite, *Toxoplasma gondii*. The parasite expresses a novel enzyme PtdThr synthase (*Tg*PTS) to produce this lipid in its endoplasmic reticulum. Genetic disruption of *Tg*PTS abrogates *de novo* synthesis of PtdThr and impairs the lytic cycle and virulence of *T. gondii*. The observed phenotype is caused by a reduced gliding motility, which blights the parasite egress and ensuing host cell invasion. Notably, the PTS mutant can prevent acute as well as yet-incurable chronic toxoplasmosis in a mouse model, which endorses its potential clinical utility as a metabolically attenuated vaccine. Together, the work also illustrates the functional speciation of two evolutionarily related membrane phospholipids, *i.e.* PtdThr and PtdSer.

This work was envisaged and initiated by myself. RDAO continued the work and performed most experiments. The study also constitutes a major part of RDAO's PhD thesis under my mentorship.

Author contributions: NG and RDAO conceived and designed the experiments; RDAO, JFB, AK, AB and IRD performed the experiments; RDAO, JFB, IRD and NG analyzed the data; RL, BJH and NG contributed reagents/materials/analysis tools; NG and RDAO wrote the paper. All authors approved the manuscript.

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Appendix K

Arunakar Kuchipudi, Ruben D Arroyo-Olarte, Friederike Hoffmann, Volker Brinkmann, Nishith Gupta
Optogenetic monitoring identifies phosphatidylthreonine-regulated calcium homeostasis in *Toxoplasma gondii*. *Microbial Cell*, 2016, 3(5): 215-23

Toxoplasma gondii is an obligate intracellular parasite, which inflicts acute as well as chronic infections in a wide range of warm-blooded vertebrates. Our recent work has demonstrated the natural occurrence and autonomous synthesis of an exclusive lipid phosphatidylthreonine in *T. gondii*. Targeted gene disruption of phosphatidylthreonine synthase impairs the parasite virulence due to unforeseen attenuation of the consecutive events of motility, egress and invasion. However, the underlying basis of such an intriguing phenotype in the parasite mutant remains unknown. Using an optogenetic sensor (gene-encoded calcium indicator, GCaMP6s), we show that loss of phosphatidylthreonine depletes calcium stores in intracellular tachyzoites, which leads to dysregulation of calcium release into the cytosol during the egress phase of the mutant. Consistently, the parasite motility and egress phenotypes in the mutant can be entirely restored by ionophore-induced mobilization of calcium. Collectively, our results suggest a novel regulatory function of phosphatidylthreonine in calcium signaling of a prevalent parasitic protist. Moreover, our application of an optogenetic sensor to monitor subcellular calcium in a model intracellular pathogen exemplifies its wider utility to other entwined systems.

This is a direct extension of previous work (Appendix J). AK, RDAO and FH performed experiments under my supervision. The work has been reported partly by RDAO in his PhD thesis and by FH in her master's research module, while AK will report the rest in his PhD work.

Author contributions: NG and RDAO conceived and designed the study; AK, RDAO and FH performed the assays; NG, AK and RDAO analyzed the data; VB and NG contributed reagents and analysis tools; NG, RDAO and AK wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix L

Ruben D Arroyo-Olarte, Nishith Gupta
Phosphatidylthreonine: An exclusive phospholipid regulating calcium homeostasis and virulence in a parasitic protist. *Microbial Cell*, 2016, 3(5): 189-190

This is a micro-review based on work mentioned in Appendix J and Appendix K. RDAO and I wrote this review following an invitation by the editorial board of the journal Microbial Cell.

Appendix M

Pengfei Kong, Christoph-Martin Ufermann, Qing Yin, Xun Suo, Bernd J Helms, Jos F Brouwers, Nishith Gupta
Two distinct CDP-diacylglycerol synthases in the parasite ER and apicoplast cooperate to ensure lipid biogenesis in *Toxoplasma gondii* (*submitted*)

Phosphatidic acid (PtdOH) serves as the universal precursor to produce all major phospholipids. In eukaryotes, PtdOH is converted into cytidine diphosphate-diacylglycerol (CDP-DAG) and diacylglycerol (DAG), which are subsequently utilized as substrates by various enzymes of lipid synthesis. Here, we report the occurrence of two phylogenetically divergent CDP-DAG synthase (CDS) enzymes in a prevalent parasitic protist *Toxoplasma gondii*. The eukaryotic-type *Tg*CDS1 is located in the endoplasmic reticulum, whereas the prokaryotic-type *Tg*CDS2 resides in the apicoplast (a plastid relict) of the parasite. While orthologs of *Tg*CDS1 appears to be conserved across the eukaryotic phyla, homologs of *Tg*CDS2 could only be found in bacteria, plants, algae and selected protozoan parasites including *Eimeria, Trypanosoma* and *Leishmania* species. Conditional knockdown of *Tg*CDS1 severely attenuated the growth of *T. gondii*, which translated into a nearly complete loss of virulence in a mouse model. Besides, mice infected with the *Tg*CDS1 mutant became categorically resistant to the challenge infection with a hypervirulent strain of *T. gondii*. The residual growth of the *Tg*CDS1 mutant was abolished by subsequent deletion of *Tg*CDS2, indicating that the host cell is unable to recompense the ablation of parasite's endogenous pathways of CDP-DAG synthesis. Lipidomics analyses of the parasite mutants revealed significant and rather specific decline in phosphatidylinositol (made in Golgi) upon genetic repression of *Tg*CDS1. Equally, deletion of *Tg*CDS2 selectively impaired the biogenesis of phosphatidylglycerol in the mitochondrion. Our data show a '*division of labor*' model of lipid biogenesis and inter-organelle trafficking, in which two discrete pools of CDP-DAG produced in the ER and apicoplast are subsequently utilized to synthesize phosphatidylinositol in the Golgi bodies and phosphatidylglycerol in the mitochondrion. Essential and divergent nature of CDP-DAG synthesis offers a validated drug target to inhibit the parasite reproduction.

This study was envisioned and directed by me. PK performed most assays. The research also constitutes PhD work of PK and the master's research module of CMU under my mentorship.

Author contributions: NG conceived the work; NG and PK designed the study; PK, CMU and QY performed the assays; NG, PK and JFB analyzed the data; BJH, JFB, XS and NG contributed reagents and analysis tools; PK and NG wrote the paper. All authors approved the manuscript.

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Appendix N

Pengfei Kong, Stefanie Brandt, Jos F Brouwers, Bernd J Helms, Richard Lucius, Nishith Gupta
Expression of exclusive phospholipids and autonomous membrane biogenesis indicate a host-independent lifestyle of *Eimeria* sporozoites (*in preparation*)

Apicomplexan parasites infect a wide range of invertebrate and vertebrate organisms and impose substantial healthcare and socioeconomic burden globally. The natural lifecycle of apicomplexan parasites involves alternating sexual an asexual reproduction occurring either in one or two hosts. The completion of sexual development is hallmarked by formation of the oocyst stage, which sporulates to produce the infective sporozoite stage for transmission to the next host. Despite its pivotal importance for inter-host transmission, the sporozoite biology remains poorly understood, primarily due to inaccessibility of this stage in sufficient amounts. In particular, the sporozoite metabolism is profoundly interesting from a conceptual viewpoint, because this is the only freely developing stage of apicomplexan parasites. It allows assessment of the 'true' metabolic potential of a parasite independent of host cell, while offering drug targets to block transmission. This work utilized *Eimeria falciformis*, which completes its entire development in mouse, and thus bestows a decent model to examine the sporozoite biology. We studied membrane biogenesis in the mature sporozoites purified from oocysts. Lipidomics analyses revealed that PtdCho (79%) and PtdEtn (13%) are the two most abundant lipids accounting for >90% of total isolated phospholipids in sporozoites, whereas PtdIns (1.5%) and PtdSer (0.5%) occur only as minor lipids. Moreover, unlike mammalian host cells, *Eimeria* sporozoites also express certain rare lipids, such as PtdThr and inositol-phosphorylceramide. We identified, experimentally-annotated and localized most of the enzymes of phospholipid biogenesis in sporozoites. In brief, we show that lipid synthesis is an evolutionary patchwork of eukaryotic and prokaryotic-type pathways located in the endoplasmic reticulum, mitochondrion, Golgi bodies, dense granules as well as apicoplast (a chloroplast relict). Collectively, this study demonstrates that the sporozoite stage is designed to be autonomous with respect to lipid biogenesis, which is a phylogenetic mosaic of inter-linked, compartmentalized and exclusive pathways. Our groundwork on membrane biogenesis of the sporozoite stage provides a sound basis to understand the network design principles of intracellular parasitism.

The study originates from our work on the membrane biogenesis of T. gondii (Appendix F-L). PK and SB, who performed most assays, will report this work in their PhD and BS theses, respectively, under my mentorship.

Author contributions: NG and PK conceived and designed the study; PK and SB performed the experiments; BJH, JFB, RL and NG contributed new reagents and analytical tools; PK, NG and JFB analyzed the data; PK and NG are preparing the manuscript.

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Appendix O

Manuela Schmid, Emanuel Heitlinger, Simone Spork, Hans P Mollenkopf, Richard Lucius, Nishith Gupta
Eimeria falciformis infection of the mouse caecum identifies opposing roles of IFNγ-regulated host pathways for the parasite development. *Mucosal Immunology*, 2014, 7(4): 969-82

Intracellular parasites reprogram host functions for their survival and reproduction. The extent and relevance of parasite-mediated host responses *in vivo* remains poorly studied, however. We utilized *Eimeria falciformis*, a parasite infecting the mouse intestinal epithelium, to identify and validate host determinants of parasite infection. Most prominent mouse genes induced during the onset of asexual and sexual growth of parasite comprise interferon γ (IFNγ)-regulated factors, *e.g.*, immunity-related GTPases (IRGA6, B6, D, M2, M3), guanylate-binding proteins (GBP2, 3, 5, 6, 8), chemokines (CxCL9-11), and several enzymes of the kynurenine pathway including indoleamine 2,3-dioxygenase 1 (IDO1). These results indicated a multifarious innate defense (tryptophan catabolism, IRG, GBP, and chemokine signaling), and a consequential adaptive immune response (chemokine-cytokine signaling and lymphocyte recruitment). The inflammation- and immunity-associated transcripts were increased during the course of infection, following influx of B cells, T cells, and macrophages to the parasitized caecum tissue. Consistently, parasite growth was enhanced in animals inhibited for CxCr3, a major receptor for CxCL9-11 present on immune cells. Interestingly, despite a prominent induction, mouse IRGB6 failed to bind and disrupt the parasitophorous vacuole, implying an immune evasion by *E. falciformis*. Furthermore, oocyst output was impaired in IFNγ-R$^{-/-}$ and IDO1$^{-/-}$ mice, both of which suggest a subversion of IFNγ signaling by the parasite to promote its growth.

I conceived the study. MS performed most of the experiments and reported this work in her PhD thesis, concluded under my supervision.

Author contributions: NG and MS designed the study; MS and SS performed the experiments; EH, HPM, and RL contributed new reagents and analysis tools; NG, MS and EH analyzed the data; MS and NG wrote the paper. All authors approved the manuscript.

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Appendix P

Manuela Schmid, Maik J Lehmann, Richard Lucius, Nishith Gupta
Apicomplexan parasite, *Eimeria falciformis*, co-opts host tryptophan catabolism for life cycle progression in the mouse. *Journal of Biological Chemistry*, 2012, 287(24): 20197-207

The obligate intracellular apicomplexan parasites, *e.g.*, *Toxoplasma gondii* and *Plasmodium* species, induce an IFNγ-driven induction of host indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism in the kynurenine pathway. Induction of IDO1 supposedly depletes cellular levels of tryptophan in host cells, which is proposed to inhibit the *in vitro* growth of auxotrophic pathogens. *In vivo* function of IDO during infections, however, is not clear, let alone controversial. We show that *Eimeria falciformis*, an apicomplexan parasite infecting the mouse caecum, induces IDO1 in the epithelial cells of the organ, and the enzyme expression coincides with the parasite development. The absence or inhibition of IDO1/2 and of two downstream enzymes in infected animals is detrimental to the *Eimeria* growth. The reduced parasite yield is not due to a lack of an immunosuppressive effect of IDO1 in the parasitized IDO1$^{-/-}$ or inhibitor-treated mice because they did not show an accentuated Th1 and IFNγ response. Noticeably, the parasite development is entirely rescued by xanthurenic acid, a by-product of tryptophan catabolism inducing exflagellation in male gametes of *Plasmodium* in the mosquito mid-gut. Our data demonstrate a conceptual subversion of the host defense (IFNγ, IDO) by an intracellular pathogen for progression of its natural life cycle. Besides, we show utility of *E. falciformis*, a monoxenous parasite of a well-appreciated host, *i.e.* mouse, to identify *in vivo* factors underlying the parasite-host interactions.

This work was envisaged by myself, and designed with the assistance of MS. MS performed most experiments. Together with Appendix O, this study constitutes a major part of MS's PhD dissertation under my mentorship.

Author contributions: NG and MS designed the study; MS and MJL performed the experiments; RL contributed new reagents/tools; MS and NG analyzed the data and wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix Q

Bingjian Ren, Manuela Schmid, Hans P Mollenkopf, Emanuel Heitlinger, Nishith Gupta
Apicomplexan parasites, *Toxoplasma gondii* and *Eimeria falciformis*, induce and co-opt a master transcription factor cFos in mammalian host cell (*in preparation*)

The protozoan phylum apicomplexa comprises more than 6000 parasite species, most of which are adapted to obligate intracellular parasitism in vertebrate and invertebrate organisms. Here, we deployed two related intracellular parasites, namely *Toxoplasma gondii* and *Eimeria falciformis*, infecting a shared host (*i.e.* mouse) to identify the host determinants of infection. Gene expression analyses of the young adult mouse colon epithelial cells infected with individual parasites revealed a notably distinct host response, indicating that irrespective of their phylogenetic proximity, both pathogens reprogram their corresponding niches in a markedly tailored manner. As expected, we detected a repertoire of host defense pathways, such as JAK-STAT, TLR and MAPK signaling and cytokine-receptor interaction, majority of which are regulated by IFNγ in response to infection. More importantly, our analyses revealed only two genes (encoding for cFos and Rab24 proteins) that were consistently induced by both parasites at early as well as late time points. Rab24 belongs to a small GTPase family likely involved in autophagy, whereas cFos is a well-known master transcription factor and a proto-oncogene, which governs diverse processes, including but not limited to, proliferation, apoptosis, inflammation and oncogenesis. In-depth phenotyping of *T. gondii* in mouse embryonic fibroblasts lacking the expression of cFos showed that the protein was needed for the parasite replication. Plaque assays using the $cFos^{-/-}$ mutant cells confirmed an apparently essential function of host cFos for the lytic cycle of *T. gondii*. Likewise, the asexual reproduction of *E. falciformis* was significantly impaired in the cFos-knockout cells. Taken together, our results signify potential subversion of a central host factor by the two intracellular parasites to promote their own development.

This is an extension of previous work (Appendix O-P). MS generated most of the datasets, which were reanalyzed and completed by BR. MS has reported a major part of this work in her PhD thesis under my supervision.

Author contributions: NG conceived the work; NG and MS designed the study; MS and BR performed the experiments; EH and HPM contributed reagents and analysis tools; MS, BR and NG analyzed the data; BR and NG are preparing the manuscript.

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Appendix R

Bilal Qureshi, Natalie E Hoffmann, Ruben D Arroyo-Olarte, Bernadette Nickl, Wolfgang Höhne, Peter R Jungblut, Richard Lucius, Patrick Scheerer, Nishith Gupta
Dynein Light Chain 8a of *Toxoplasma gondii*, a unique conoid-localized β-strand-swapped homodimer, is required for an efficient parasite growth. *FASEB J*, 2013, 27(3): 1034-47

Dynein light chain 8 (DLC8) is a ubiquitous eukaryotic protein regulating diverse cellular functions. We show that the obligate intracellular parasite *Toxoplasma gondii* harbors 4 DLC8 proteins (*Tg*DLC8a-d), of which only *Tg*DLC8a clusters in the mainstream LC8 class. *Tg*DLC8b-d proteins form a divergent and alveolate-specific clade. *Tg*DLC8b-d proteins are largely cytosolic, whereas *Tg*DLC8a resides in the conoid at the apical end of *T. gondii*. The apical location of *Tg*DLC8a is also not shared by its nearly identical *Eimeria* (*Et*DLC8a), *Plasmodium* (*Pf*DLC8), or human (*Hs*DLC8) orthologs. Notwithstanding an exclusive conoid targeting, *Tg*DLC8a exhibits a classical LC8 structure. It forms a homodimer by swapping of the β strands that interact with the antiparallel β' strands of the opposing monomers. The *Tg*DLC8a dimer contains two identical binding grooves and appears to be adapted for multitarget recognition. By contrast, the previously reported *Pf*DLC8 homodimer is shaped by binding of the β strand with the parallel β' strand and lacks such a distinct binding interface. Our comparisons suggest an unexpected structural and functional divergence of the two otherwise conserved proteins from apicomplexan parasites. Finally, we demonstrate that a phosphomimetic S88E mutation renders the *Tg*DLC8a-S88E mutant monomeric and cytosolic in *T. gondii*, and its overexpression inhibits the parasite growth in human fibroblasts.

The work was conceived by me, and directed together with PS. The study was designed with the support of BQ and PS. BQ and NEH performed most assays, aided or facilitated by RDAO and BN. BQ and NEH have reported this work in their master's thesis under my supervision.

Author contributions: NG conceived the study; NG, BQ and PS designed the work; BQ, NEH, BN and RDAO performed the assays; RL, WH and PRJ contributed new reagents and analysis tools; NG, BQ and PS analyzed the data and wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix S

Anne Hartmann, Ruben D Arroyo-Olarte, Katharina Imkeller, Peter Hegemann, Richard Lucius, Nishith Gupta
Optogenetic modulation of an adenylate cyclase in *Toxoplasma gondii* demonstrates a requirement of parasite cAMP for host-cell invasion and stage differentiation. *Journal of Biological Chemistry*, 2013, 288(19): 13705-17

Successful infection and transmission of the obligate intracellular parasite *Toxoplasma gondii* depends on its ability to switch between fast-replicating tachyzoite (acute) and quiescent bradyzoite (chronic) stages. Induction of cAMP in the parasitized host cells has been proposed to influence parasite differentiation. It is not known whether the parasite or host cAMP is required to drive this phenomenon. Other putative roles of cAMP for the parasite biology also remain to be identified. Unequivocal research on cAMP-mediated signaling in such intertwined systems also requires a method for an efficient and spatial control of the cAMP pool in the pathogen or in the enclosing host cell. We have resolved these critical concerns by expressing a photoactivated adenylate cyclase that allows light-sensitive control of the parasite or host-cell cAMP. Using this method, we reveal multiple roles of the parasite-derived cAMP in host-cell invasion, stage-specific expression, and asexual differentiation. An optogenetic method provides many desired advantages, such as (i) rapid, transient and efficient cAMP induction in extracellular/intracellular and acute/chronic stages; (ii) circumvention of the difficulties often faced in cultures, *i.e.* poor diffusion, premature degradation, steady activation, and/or pleiotropic effects of cAMP agonists and antagonists; (iii) genetically encoded enzyme expression, thus inheritable to the cell progeny; and (iv) conditional and spatiotemporal control of cAMP levels. Importantly, a successful optogenetic application in *Toxoplasma* also illustrates its wider utility to study cAMP-mediated signaling in other genetically amenable two-organism systems, such as in symbiotic and pathogen-host models.

I conceived and designed the study, and supervised AH and RDAO (PhD students), who equally contributed to the results. The work was partly assisted by a master's student KI.

Author contributions: NG conceived and designed the work; AH, RDAO and KI performed the experiments; RL and PH contributed new reagents and analytical tools; NG, AH and RDAO analyzed the data; NG, AH and RDAO wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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Appendix T

Tatsuki Sugi, Yan Fen Ma, Tadakimi Tomita, Fumi Murakoshi, Michael S Eaton, Rama Yakubu, Bing Han, Vincent Tu, Kentaro Kato, Shin-Ichiro Kawazu, Nishith Gupta, Elena S Suvorova, Michael W White, Kami Kim, Louis M Weiss
Toxoplasma gondii cAMP-dependent protein kinase subunit 3 is involved in the switch from tachyzoite to bradyzoite development. *mBio*, 2016, 7(3): e00755-16

Toxoplasma gondii is an obligate intracellular apicomplexan parasite that infects warm-blooded vertebrates, including humans. Asexual reproduction in *T. gondii* allows it to switch between the rapidly replicating tachyzoite and quiescent bradyzoite life cycle stages. A transient cyclic AMP (cAMP) pulse promotes bradyzoite differentiation, whereas a prolonged elevation of cAMP inhibits this process. We investigated the mechanism(s) by which differential modulation of cAMP exerts a bidirectional effect on parasite differentiation. There are three protein kinase A (PKA) catalytic subunits (*Tg*PKAc1-3) expressed in *T. gondii*. Unlike *Tg*PKAc1 and *Tg*PKAc2, which are conserved in the phylum Apicomplexa, *Tg*PKAc3 appears evolutionarily divergent and specific to coccidian parasites. *Tg*PKAc1 and *Tg*PKAc2 are distributed in the cytomembranes, whereas *Tg*PKAc3 resides in the cytosol. *Tg*PKAc3 was genetically ablated in a type II cyst-forming strain of *T. gondii* (PruΔ*ku80*Δ*hxgprt*) and in a type I strain (RHΔ*ku80*Δ*hxgprt*), which typically does not form cysts. The Δ*pkac3* mutant exhibited slower growth than the parental and complemented strains, which correlated with a higher basal rate of tachyzoite-to-bradyzoite differentiation. 3-Isobutyl-1-methylxanthine (IBMX) treatment, which elevates cAMP levels, maintained wild-type parasites as tachyzoites under bradyzoite induction culture conditions (pH 8.2/low CO_2), whereas the Δ*pkac3* mutant failed to respond to the treatment. This suggests that *Tg*PKAc3 is the factor responsible for the cAMP-dependent tachyzoite maintenance. In addition, the Δ*pkac3* mutant had a defect in the production of brain cysts in vivo, suggesting that a substrate of *Tg*PKAc3 is probably involved in the persistence of this parasite in the intermediate host animals.

I have not been involved in conceiving, designing and performing this work, which was predominantly a cumulative effort of LMW, KK and TS. I have played an advisory role, contributed analytical tools and edited the manuscript.

Author contributions: LMW, KK and TS conceived and designed the work; TS, YFM, TT, FM, MSE, RY, BH and VT performed the experiments; KK, SIK, NG, ESS and MWW contributed reagents and analytical tools; TS, LMW and KK analyzed the data and wrote the paper. All authors approved the manuscript.

All necessary declarations pertaining to this research (acknowledgments, conflict of interests, data deposition *etc.*) have been made by all coauthors during the publication of this work.

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6 ACKNOWLEDGMENT

It is already 13 years since I turned to the long winding path of molecular parasitology, and there has been no look back since then. I am still learning to drive despite the fact that many excellent mentors have invested an ample amount of time and effort to nurture me. I wish to profoundly thank Richard Lucius, who has been an excellent guide and a wonderful adviser since April 2006. It is in fact a Herculean task for me to describe the number of occasions when I needed him most. Literally speaking, he charmed me to drive in Berlin from the mile-high city Denver. He provided me a decent car (laboratory), gasoline (seed funds), upkeep staff (technician) and the freedom to venture on a racetrack (research program) of my own choice. Having driven in the United States, I was pampered with cheap gasoline (superfluous research funds), lenient traffic rules (ease of doing work) and wider roads (diversity of research and interactions). It was not easy to adjust, improve and sustain my driving skills while training the next generation of drivers (young scientists). But guess what… he was always there. He frequently responded to my queries in off-hours, giving me a feeling that he truly cared for me. His relentless support has often gone beyond science. For instance, he helped me to build my personal life and family in Germany. Can never appreciate him enough in words. Thank you very much RICHARD.

Next one to thank is Dennis Voelker, my postdoctoral mentor (and now collaborator), who gave me the very first opportunity to start driving in the terrain of molecular parasitology. Not knowing much about how well I would fare with parasites, it was indeed not easy to switch fields after my PhD in Biochemistry. I was clearly not aware of the pitfalls and potholes along the road. Ironically, Dennis was also venturing first time in this territory. Nonetheless, some primary traffic rules and rather uncommon commonsense (the art of doing science) learnt from Dennis enabled me to spearhead a new territory. I am deeply inspired by his way of doing science, where consistent and gold standard performance matter more than bumpy rides. Other valuable tenets, I picked up from him are '*maliciousness is simply unfamiliarity with things*', and '*the absence of evidence doesn't mean the evidence of absence*'. Then, there are two more coaches, Kai Matuschewski and Peter Hegemann, who greatly improved my driving skills by offering much-needed advices, hardcore support and recommendations when I needed. Each and every meeting I had with them steered me into the right direction, and often rekindled my mind to think beyond the obvious. Thank you very much DENNIS, KAI and PETER for your continued support.

I am also obliged to Hermann-Georg Holzhütter and Andreas Herrmann, co-applicants in the first two grants of mine, which were indispensable to kick-start a long-drive. Even though I never quite fathomed their research fields (system biology, biophysics), I did learn two must-learn tacts: writing grants and being interdisciplinary. Then there are several collaborators and cooperation partners, particularly Isabelle Coppens, Scott Landfear, Dominique Soldati-Favre, Bernd Helms, Jos Brouwers, Hans Mollenkopf, Wolfgang Höhne, Ildiko Dunay, Vyacheslav Zagoriy, Volker Brinkmann and Emanuel Heitlinger, all of whom have reinforced our work by contributing complementary expertise. I am also very indebted to many facilitators, Boris Striepen, John Boothroyd, Vern Carruthers, Markus Meissner, David Bzik, Peter Bradley, Sergio Angel, Louis Weiss and Jean-François Dubremetz, who have kept our automobile running by donating precious spare parts (biological resources) at their earliest possible convenience. I want to pay my special gratitude to Grit Meusel, our vital upkeep staff. I tenderly call her the '*mother of our lab'*, which is quite the right description for an important person like her. No one notices the people changing wheels and spare parts when the car racer is on the racetrack. But such behind-the-

scene personnel are actually the lynchpin in any discipline, be it science or Hollywood Sci-Fi. I also take this opportunity to acknowledge two other colleagues in the department, Susanne Hartmann and Maik Lehmann for their occasional active support.

Now that I have my own driving school for the next-generation drivers (lab members), it is worth saluting what I have learnt while training them. A series of PhD, MS and BS students including Martin Blume, Manuela Schmid, Vera Sampels, Ruben Dario Arroyo-Olarte, Anne Hartmann, Pengfei Kong, Richard Nitzsche, Arunakar Kuchipudi, Matthias Noll, Ozlem Günay, Laura Radtke, Bingjian Ren, Stefanie Brandt, Theresa Ring, Julian Kreibich, Aline Hössler, Leska Balken, Fatima Hedar, Lucas Niedersen, Ludmila Lobkowicz, Isabelle Dietrich, Annabell Bachem, Bilal Qureshi, Clemens Falker, Natalie Hofmann, Ulrich Sternberg, Stephan Marquardt, Maximilian Tischer, Tobias Kletter, Maria Hellmund, Katharina Imkeller, Stefania Chiocchetti, Nicola Schaltenberg, Friederike Hoffmann, Bernadette Nickl, Ines Heyn, Christina Wangen, Jennifer Oduro, Marjorie Linares, Kathrin Frenzel, Christoph-Martin Uffermann, Diana Zimmermann, Mareen Lüthen and Julieta Cuellar, all of whom worked really hard (and smart) to get my car going for a long drive. Going along the way, they came up with own ideas and maneuvers to get going further. Assimilating and reconciling their concepts with mine was not always easy, particularly while maintaining the course (overarching research theme). One very good trait, I have become used to by now is: *When you change the way you look at things, things you look at, change*. They have also transformed me into a disciplined, organized (as well as humble) character with loads of other abilities required in the world of science.

Outside workplace, my earnest appreciation goes to my beloved wife Alka and little ones, Aryan, Nitya (and Aayra), for their constant reinforcement, inspiration and cooperation. Even though it sounded a bit weird when Aryan and Nitya at the age of 4 and 5 years said, '*Daddy, you are getting late for your office*', pushing me out; and '*Daddy, you need to work more than Richard Lucius*', when I worked at home; it was an inspirational push I cherished every day. Alka, for one, has been one of the most central persons in my life for the last 20 years, categorically resigning her own dreams to support my carrier and build our family in Germany, despite the fact that her heart and mind are still in India. Caring for kids during the school and kindergarten holidays, as well as during ailment periods and business travels, she bestowed me myriad extra hours of work, for which I can never thank her enough. I also sincerely acknowledge my Parents and Family Members in India, who have been a major brute force to back my carrier in science. Then there were many festive as well as tragic occasions when they all had to put up with my unforeseen absence due to academic obligations. Work and Life, both would have been agonizing without your backing.

Second last, I wish to express my sincere gratitude to various funding agencies. This work would never have been realized without generous funds from many funding sources, mainly German Research Foundation (DFG), Humboldt University, Helmholtz Foundation, International Max-Planck Research School, National Institute of Health, European Society of Clinical Microbiology and Infectious Diseases, European Molecular Biology Organization, Federation of European Biochemical Societies and Novartis Animal Health. Last but not least, I am extremely grateful to you as a Reader for your patience and time to read this piece of work, which will navigate you through my last 13 years of journey. I want to apologize for any sections of this work, which may sometime be challenging or perplexing to a non-expert. The excuse is obvious: *my voyage through science to solve the great mystery of intracellular parasitism is still on*.

7 CURRICULUM VITAE

AWARDS & HONORS

Nov 2015	Young Scientist Award in Microbiology by Robert Koch Foundation (Germany)
Feb 2015	Indian National Science Academy (INSA) Fellow (University of Delhi, India)
July 2014	Carl Asmund Rudolphi Medal by the German Society of Parasitology (Germany)
August 2009	Chinese Academy of Science Fellow (Peking University Medical College, China)

FELLOWSHIPS/GRANTS

2016 – 2021	Heisenberg Fellowship by German Research Foundation, Germany
2006 – 2008	Postdoctoral Fellowship from German Research Foundation (DFG)
2003 – 2006	Postdoctoral Fellowship from National Institute of Health (NIH), USA
1999 – 2003	PhD Fellowship from German Research Foundation (DFG)
1997 – 1999	MS-Biotech Stipend from Ministry of Science, Government of India
2000 – Present	Travel awards from EMBO, FEBS, ESCMID, IUBMB, WAAVP, COST857, DAAD, DFG, GSK, ASM, GRC, ASBMB, SLAS, BS-UK and GBM

EXTERNAL FUNDING

2015 – 2018	Optogenetic dissection of cyclic NMP signaling in *T. gondii*: GU 1100/7-1 from German Research Foundation, 197.550€
2015 – 2019	Cyclic NMP signaling and metabolic regulation in *T. gondii*: GRK2046/A2 from German Research Foundation, 133.272€
2014 – 2017	Synthesis *vs.* import of host-derived phospholipids by *T. gondii*: GU1100/4-1 from German Research Foundation; co-PI: Maik Lehmann, 319.900€
2013 – 2016	Carbon metabolisms of acute and chronic stages of *T. gondii*: GU1100/3-1 from German Research Foundation, 159.150€
2010 – 2012	Phosphatidylcholine synthesis as a drug target in *T. gondii*: Young scientist grant from European Society of Infectious Diseases and Microbiology, 20.000€
2009 – 2014	Biogenesis of inositol-lipids in *T. gondii*: GRK1121/A7 from German Research Foundation, co-PI: Andreas Herrmann (HU, Berlin), 181.000€
2009 – 2013	Modeling of lipid biogenesis in *Toxoplasma*-infected human cell SBF618/C7 from German Research Foundation, co-PIs: Herrmann Holzhütter (Charité, Berlin) and Richard Lucius (HU, Berlin), 410.700€
2009 – 2011	High-throughput anti-coccidian drug screening using YFP-parasite Novartis Animal Healthcare, co-PI: Richard Lucius (HU, Berlin), 32.725€
2009 – 2011	*In vivo* parasite-host interactions in the *Eimeria*-mouse model Awarded to Manuela Schmid and Nishith Gupta/Richard Lucius (Host labs at HU, Berlin) by Helmholtz Foundation, Germany, ~120.000€
2008 – 2010	Central carbon metabolism of *T. gondii* Awarded to Martin Blume and Nishith Gupta/Richard Lucius (Host labs at HU, Berlin) by Helmholtz Foundation, Germany, ~120.000€

STUDENT TEACHING

2009 – Present	Summer Semester module for Master students (MB-A05): Biochemistry and Cell Biology of Parasites 14 lectures, 2-weeks practical course and journal club, Organized jointly with Kai Matuschewski (Humboldt University, Berlin)
2010 – Present	Winter Semester module for Master students (MB-A04): Molecular Manipulation of Parasites, 2-weeks practical course, Humboldt University, Berlin

ORGANIZATION SKILLS

2008	Pathogen-Host-Interplay, International Summer School (Berlin) Funding: FEBS, DAAD and ESCMID, Co-organizer: Martina Sick Volume: 52.000 Euros plus 15 International Travel Fellowships
2007	Pathogen-Host-Interplay, International Summer School (Berlin) Funding: Center of Infection and Immunity (ZIBI), Co-organizer: Martina Sick
2006 – 2008	International Colloquiums in Infection Biology for PhD students (Berlin) Funding: Center of Infection and Immunity (ZIBI)

MENTORSHIP

PhD students

2015 – Present	Bingjian Ren: CRISPR/Cas9-assisted reverse genetics in *E. falciformis*
2015 – Present	Laura Radtke: Stage-specific metabolism and signaling in *T. gondii*
2015 – Present	Ozlem Günay: Genetic dissection of cGMP signaling in *T. gondii*
2014 – Present	Matthias Noll: Optogenetic dissection of cAMP signaling in *T. gondii*
2014 – Present	Arunakar Kuchipudi: Lipid-regulated calcium homeostasis in *T. gondii*
2012 – 2016	Richard Nitzsche: Central carbon metabolism of *T. gondii*
2012 – 2016	Pengfei Kong: Synthesis and roles of phospholipids in *T. gondii* and *E. falciformis*
2010 – 2014	Anne Hartmann: Phosphatidylethanolamine biogenesis in *T. gondii*
2009 – 2014	Ruben Dario Arroyo-Olarte: Phosphatidylthreonine biogenesis in *T. gondii*
2009 – 2012	Manuela Schmid: Host determinants of *Eimeria* development in mouse model
2009 – 2011	Vera Sampels: Phosphatidylcholine biogenesis in *T. gondii*
2008 – 2010	Martin Blume: Sugar metabolism of *T. gondii* and *P. berghei*

MS, BS and Internship students (on topics related to aforementioned PhD students)

2006 – Present	Lisa-Elena Pfluger, Diana Zimmermann, Mareen Lüthen, Julieta Cuellar, Stefanie Brandt, Theresa Ring, Laura Radtke, Julian Kreibich, Aline Hössler, Leska Balken, Fatima Hedar, Lucas Niedersen, Ludmila Lobkowicz, Maximilian Tischer, Ulrich Sternberg, Heidy Narvaez, Tobias Kletter, Christoph Uffermann, Stefania Chiocchetti, Maria Hellmund, Nicola Schaltenberg, Friederike Hoffmann, Katharina Imkeller, Bilal Qureshi, Natalie Hofmann, Bernadette Nickl, Clemens Falker, Stephan Marquardt, Ines Heyn, Christina Wangen, Jennifer Oduro, Marjorie Linares, Kathrin Frenzel, Manuela Schmid, Isabelle Dietrich, Annabell Bachem, Martin Blume

KEY PRESENTATIONS

April 2016	Make it or take it – Phospholipid biogenesis in *Toxoplasma gondii* Infection Immunology Meets Molecular Microbiology, Erlangen, Germany
June 2015	Phosphatidylthreonine and lipid-mediated control of parasite virulence Toxoplasmosis Conference, Gettysburg, USA
Jan/Feb 2015	A lethal intimacy – Metabolic basis of parasite-host interplay and infidelity IIS and NCBS (Bangalore), CDRI (Lucknow), JNU (New Delhi), India
Nov 2014	Optogenetics-mediated regulation of cAMP signaling in *Toxoplasma gondii* International Workshop on Opportunistic Protists, Seville, Spain
Nov 2014	Metabolic basis of parasite-host interplay and infidelity Veterinary University of Madrid, Spain
May 2014	Metabolic basis of obligate intracellular parasitism University of Edinburgh, Scotland, UK
April 2014	Opposing functions of the mouse IFNγ signaling during *Eimeria* infection International Congress on Infectious Diseases, Cape Town, South Africa
November 2013	Make it or take it – Lipid biogenesis in *Toxoplasma gondii* University of Utrecht, The Netherlands
November 2013	Opposing roles of IFNγ signaling during *Eimeria* infection Apicowplexa Meeting, Kusadasi, Turkey
September 2012	Optogenetic control of cytosolic cAMP in *Toxoplasma gondii* EMBO Meeting, Nice, France
December 2010	*Toxoplasma gondii*: A model organism to explore pathogen-host interactions University of Veterinary Medicine, Vienna, Austria
August 2010	Phospholipid biogenesis in *Toxoplasma gondii* International Congress of Parasitology (ICOPA), Melbourne, Australia
August 2009	*Toxoplasma gondii* secretes a soluble PtdSer decarboxylase World Association for Veterinary Parasitology (WAVP), Calgary, Canada
June 2008	Membrane biogenesis in *Toxoplasma gondii* COST857 Workshop on Apicomplexan Parasites, Crete, Greece
December 2006	Membrane biogenesis in *Toxoplasma gondii* EMBO Course on RNAi in *Trypanosoma brucei*, Nairobi, Kenya

MEMBERSHIPS

2015 – Present	Robert Koch Foundation, Germany
2014 – Present	German Society of Parasitology (DGP), Germany
2001 – Present	Biochemical Society (FEBS constituent), United Kingdom
2007 – Present	European Society of Clinical Microbiology and Infectious Diseases (ESCMID)
2007 – Present	International Society of Infectious Diseases (ISID)
2000 – 2008	Society of Biochemistry and Molecular Biology (FEBS constituent), Germany

OTHER INFORMATION

5-yrs Citations	656 (Source: Google Scholar)
Editorial Board	Microbial Cell
Journal Reviewer	Elsevier, FEBS, FEMS, JBC, PLoS Journals, Wiley, ASM
Grant Reviewer	MRC, BBSRC, Welcome Trust – DBT
Feature Article	1) Exploiting the Host: *International Innovation* Magazine Sept 2013 2) Optogenetics in infection research (*due in* 2018)
Patents	Genetically attenuated vaccine against *Toxoplasmosis gondii* and commercial usage of phosphatidylthreonine (EP20846-Ro/td, WO23005Ro/td; in *review*)

PUBLICATIONS

(1) Sugi T, Ma YF, Tomita T, Murakoshi F, Eaton MS, Yakubu R, Han B, Tu V, Kato K, Kawazu SI, **Gupta N**, Suvorova ES, White MW, Kim K, Weiss LM (2016) *Toxoplasma gondii* cAMP-dependent protein kinase subunit 3 is involved in the switch from tachyzoite to bradyzoite development. *mBio*, 7(3): e00755-16

(2) Kuchipudi A, Arroyo-Olarte RD, Hoffmann F, Brinkmann V, **Gupta N** (2016) Optogenetic monitoring of the parasite calcium identifies a phosphatidylthreonine-regulated ion homeostasis in *Toxoplasma gondii*. *Microbial Cell*, 3(5), 215-23

(3) Arroyo-Olarte RD and **Gupta N** (2016) Phosphatidylthreonine: an exclusive phospholipid regulating calcium homeostasis and virulence in a parasitic protest. *Microbial Cell*, 3(5): 189-90

(4) Nitzsche R, Zagoriy V, Lucius R, **Gupta N** (2016); Metabolic cooperation of glucose and glutamine is essential for the lytic cycle of obligate intracellular parasite *Toxoplasma gondii*. *Journal of Biological Chemistry*, 291(1): 126-41

(5) Arroyo-Olarte RD, Burrowers JF, Kuchipudi A, Helms JB, Biswas A, Dunay IR, Lucius R, **Gupta N** (2015); Phosphatidylthreonine and lipid-mediated control of parasite virulence. *PLoS Biology,* 13 (11): e1002288

(6) Blume M, Nitzsche R, Sternberg U, Gerlic M, Masters SL, **Gupta N**, McConville MJ (2015); A *Toxoplasma gondii* gluconeogenic enzyme contributes to robust central carbon metabolism and is essential for replication and virulence. *Cell Host & Microbe*, 18(2): 210-20

(7) Hartmann A, Hellmund M, Lucius R, Voelker DR, **Gupta N** (2014); Phosphatidylethanolamine synthesis in the parasite mitochondrion is required for efficient growth but dispensable for survival of *Toxoplasma gondii*. *Journal of Biological Chemistry*, 289(10): 6809-24

(8) Schmid M, Heitlinger E, Spork S, Mollenkopf HP, Lucius R, **Gupta N** (2014); *Eimeria falciformis* infection of the mouse caecum identifies opposing roles of IFNγ-regulated host pathways for the parasite development. *Mucosal Immunology*, 7(4): 969-82

(9) Hartmann A, Arroyo-Olarte RD, Imkeller K, Hegemann P, Lucius R, **Gupta N** (2013); Optogenetic modulation of an adenylate cyclase in *Toxoplasma gondii* demonstrates a requirement of parasite cAMP for host-cell invasion and stage differentiation. *Journal of Biological Chemistry*, 288(19): 13705-17

(10) Qureshi B, Hoffmann N, Arroyo-Olarte RD, Nickl B, Höhne W, Jungblut P, Lucius R, Scheerer P, **Gupta N** (2013); Dynein Light Chain 8a of *Toxoplasma gondii*, a unique conoid-localized β-strand-swapped homodimer, is required for an efficient parasite growth *FASEB J*, 27(3): 1034-47

(11) **Gupta N**, Hartmann A, Lucius R, Voelker DR (2012) The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase. *Journal of Biological Chemistry*, 287(27): 22938-47

(12) Schmid M, Lehmann MJ, Lucius R, **Gupta N** (2012) Apicomplexan parasite, *Eimeria falciformis*, co-opts host tryptophan catabolism for life cycle progression in the mouse. *Journal of Biological Chemistry*, 287(24): 20197-207

(13) Sampels V, Hartmann A, Dietrich I, Coppens I, Sheiner L, Striepen B, Herrmann A, Lucius R, **Gupta N** (2012) Conditional mutagenesis of a novel choline kinase demonstrates the plasticity of phosphatidylcholine biogenesis and gene expression in *Toxoplasma gondii*. *Journal of Biological Chemistry*, 287(20): 16289-99

(14) Blume M, Hliscs M, Contreras D, Sanchez M, Landfear S, Lucius R, Matuschewski K, **Gupta N** (2011) A constitutive pan-hexose permease in *Plasmodium* and models for high-throughput screening of anti-malarial sugar analogs. *FASEB J*, 25(4): 1218-29

(15) Shao D, Liu Y, Liu X, Zhu L, Cui Y, Cui A, Qiao A, Kong X, Liu Y, Chen Q, **Gupta N**, Fang F, Chang Y (2010) PGC-1β-regulated mitochondrial biogenesis and function in myotubes is mediated by NRF-1 and ERRα. *Mitochondrion*, 10(5): 516–27

(16) Zhu LL, Liu Y, Cui AF, Shao D, Liang JC, Liu XJ, Chen Y, **Gupta N**, Fang FD, Chang Y (2010) PGC-1α coactivates estrogen receptor α to induce the expression of glucokinase. *American Journal of Physiology Endocrinology Metabolism*, 298(6): E1210–8

(17) Kong X, Fan H, Liu X, Wang R, Liang J, **Gupta N**, Chen Y, Fang F, Chang Y (2009) Peroxisome proliferator-activated receptor gamma coactivator-1alpha enhances antiproliferative activity of 5'-deoxy-5-fluorouridine in cancer cells through induction of uridine phosphorylase. *Molecular Pharmacology*, 76(4): 854-60

(18) Blume M, Contreras DR, Landfear S, Fleige T, Soldati DF, Lucius R, **Gupta N** (2009) Host-derived glucose and its transporter in the obligate intracellular pathogen *Toxoplasma gondii* are dispensable by glutaminolysis. *Proceedings of National Academy of Sciences USA*, 106(31): 12998–3003

(19) Liu X, Feng Q, Chen Y, Zuo J, **Gupta N**, Chang Y, Fang F (2009) Proteomics-based identification of differentially-expressed proteins including galectin-1 in the blood plasma of type 2 diabetic patients. *Journal of Proteome Research*, 8(3): 1255-62

(20) Jiang ZB, Song HT, **Gupta N**, Ma LX, Wu ZB (2007) Cell surface display of functionally active lipases from Yarrowia lipolytica in *Pichia pastoris*. *Protein Expression Purification*, 56(1): 35-9

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8 DECLARATIONS (ERKLÄRUNG)

(a) Ich versichere hiermit an Eidesstatt, dass ich die Habilitationsschrift bzw. eine Mehrzahl von Fachpublikationen mit dem einer Habilitationsschrift entsprechenden wissenschaftlichen Gewicht selbständig angefertigt habe.

(b) Hiermit erkläre ich, dass anderweitig kein Habilitationsverfahren durchgeführt wird. Falls bereits ein Habilitationsverfahren durchgeführt wurde, finden Sie die vollständigen Angaben über dessen Ausgang auf einem gesonderten Blatt.

(c) Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Habilitationsordnung der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin vom 21. März 2016.

(d) Weiterhin erkläre ich, dass die Regeln der guten wissenschaftlichen Praxis, wie sie in der „Satzung der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis und über den Umgang mit Vorwürfen wissenschaftlichen Fehlverhaltens“ in der jeweils geltenden Fassung festgelegt sind, eingehalten wurden.

(e) Ich erkläre hiermit, dass ein Strafverfahren beim Gericht nicht anhängig ist.

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Datum und Unterschrift der Kandidatin/des Kandidaten

9 PUBLICATIONS (REPRESENTATIVE FULL-TEXT ARTICLES)

Following pages contain the full text of selected original research articles partly embodying this work. Other full-text publications with supplementary datasets can be accessed at:

https://www.ncbi.nlm.nih.gov/pubmed/?term=Nishith+Gupta

https://www.researchgate.net/profile/Nishith_Gupta